THE BIOCHEMICAL JOURNAL

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[16-^{3}H]Progesterone Metabolism in Advanced Pregnancy and in Oophorectomized–Hysterectomized Women

By W. H. PEARLMAN
Guy's Hospital Medical School, London, S.E. 1

(*Received* 27 *December* 1956)

The percentage recovery of administered progesterone as urinary pregnanediol has been reported to be much higher in pregnancy than in the non-pregnant state in the absence of luteal function (Venning & Browne, 1940; Sommerville & Marrian, 1950; Guterman, 1953). (The term 'pregnanediol' as used by some of the authors consisted largely, but not necessarily entirely, of 5β-pregnane-3α:20α-diol.) The extent of this metabolic conversion has similarly been related to the maintenance of pregnancy in patients with threatened abortion (Guterman, 1953). The validity of these observations might bear re-examination—indeed, the data on normal pregnancy could not be confirmed recently (Klopper & Michie, 1956)—in view of the crude, albeit useful, analytical methods employed in the early pioneer investigations; also, the observations of Sommerville & Marrian (1950) were subsequently revised (Marrian, Russell & Atherden, 1954). The determination of the additional amount of pregnanediol in pregnancy urine which arises from administered progesterone is subject to considerable error because (*a*) the control or base-line values for urinary pregnanediol fluctuate from day to day and progressively increase with advancing pregnancy (Venning, 1948), and (*b*) unless very large amounts of progesterone are administered, there is only a small increase in the urinary pregnanediol excretion. These difficulties may be obviated by administering isotopically labelled hormone (such as [16-^{3}H]progesterone) and a reinvestigation of the problem was accordingly undertaken; more information on progesterone metabolism was also expected with the isotope-tracer technique.

EXPERIMENTAL

Materials

[16-^{3}H]Progesterone (nominal specific activity 22·2 μc/mg.) was prepared in this Laboratory (Pearlman, 1957). A solution of the radioactive hormone in arachis oil was sterilized by heating at 120° for 1 hr.

Determination of tritium

By combustion of the sample to water, conversion into tritium–hydrogen gas, and gas counting. The procedures described by Avivi, Simpson, Tait & Whitehead (1954) were used. This time-consuming method was reserved chiefly for the analysis of the crude urinary ketonic and non-ketonic fractions.

By liquid scintillation counting. The apparatus was essentially that described by Avivi *et al.* (1954). The sample was dissolved in toluene (1·5 ml.) containing 0·5% (w/v) of the phosphor, *p*-terphenyl; the detection efficiency for counting tritium was about 7% and a reference standard of a steroid of known specific activity was employed on each occasion. This method was employed in the analysis of purified crystalline preparations or of colourless or only slightly coloured neutral urinary fractions. It was unsuitable for the accurate analysis of the crude urinary ketonic and non-ketonic fractions because of the presence of pigmented and fluorescent material. The isotope values for such fractions deviated by about $\pm$20% from those established by the combustion procedure. An attempt to introduce a correction by adding a known amount of colourless radioactive steroid to the crude urinary fractions was unsuccessful.

By counting in a windowless gas-flow counter. A few samples of high specific activity were analysed in a windowless counter (Tracerlab. Inc., Boston, Mass., U.S.A.) according to an unpublished procedure devised by Dr J. F. Tait and co-workers at the Middlesex Hospital Medical School, London. The sample (20 μg. or less of solids) was plated on a flat planchet containing many concentric grooves in order to promote uniform distribution of material over an area of 5 cm.^{2}. The count was corrected for self-absorption after the addition of a steroid standard of very high specific activity and negligible weight. The isotope values thus obtained were in good agreement with those established by the combustion method.

Administration of hormone and isolation of metabolic products from urine

After intramuscular injection of [16-^{3}H]progesterone as a single dose in each case, the urine was collected for 4 days (Expts. 1 and 2, Table 1) or 6 days (Expts. 3–5, Table 1), with toluene as preservative. The urinary excretion of isotope appeared to be practically complete by the end of the fourth day: thus, in Expt. 3, of the total amount of isotope eliminated as neutral material, 65, 22 and 13% appeared in the first, second and third 48 hr. collection periods; in Expt. 4 the corresponding figures were 60, 30 and 9% respectively.

The urine was usually worked up immediately, but in a few instances the specimens were stored in the refrigerator for 1 or 2 days. The urine specimen (for example, 1000 ml.) was brought to the boil and 10N-HCl (100 ml., A.R. quality) was cautiously added through a condenser;

refluxing was continued for exactly 10 min. and the solution was rapidly cooled. The hydrolysate was extracted with peroxide-free ether (1×1000 ml.; 2×500 ml.). The ether extract was washed with water (4×50 ml.) and evaporated. The residue was further purified by partitioning between benzene (100 ml.) and N-NaOH (4×50 ml.); with the pregnancy urines, the volumes of benzene and N-NaOH were larger in order to effect complete solution of the semi-crystalline residue (crude pregnanediol) which was obtained at this stage. The benzene phase was freed from alkali by repeated washing with water and then evaporated to dryness. The residue constituted the neutral fraction. It was partitioned into ketonic and non-ketonic moieties with the aid of Girard's reagent T (Girard & Sandulesco, 1936). A small amount of ketonic material was removed from the non-ketonic fraction on further treatment with Girard reagent; the ketonic products were combined and similarly retreated to give the final ketonic fraction. The tritium content (Table 1) of the final ketonic and non-ketonic fractions was determined on small aliquot portions.

The non-ketonic fraction was acetylated with pyridine–acetic anhydride (2:1, v/v, sufficient to effect solution on warming); after 48 hr. at room temperature the solution was diluted with water and extracted three times with ether. The ether extract, after washing successively with small portions of 2N-HCl (five times), 5% (w/v) Na_2CO_3 (twice), and water (four times), was evaporated. With the non-pregnancy non-ketonic fractions, to an aliquot of which inert 5β-pregnane-3α:20α-diol had been added before acetylation, the final acetylated product weighed less than 40 mg. It was dissolved in about 0·5 ml. of dry peroxide-free ether (stored over Na wire and redistilled over CaH_2 just before use) and 2 ml. of light petroleum (AnalaR, b.p. below 40°, treated with conc. H_2SO_4 in a separating funnel, carefully washed with water, dried over CaH_2 and distilled). The solution was filtered on a column (8 mm. $\times$ 80 mm.) containing 3·0 g. of Al_2O_3 (Type H, Peter Spence and Sons Ltd., Widnes, previously treated with acetic acid, exhaustively washed with water and dried at 150° overnight). Elution was made with four 10 ml. portions of light petroleum and finally with 20 ml. of dry ether. The crystalline solids from light-petroleum eluates were repeatedly recrystallized from ether–light petroleum or from aqueous methanol to give 5β-pregnane-3α:20α-diol 3:20-diacetate (3α:20α-diacetoxy-5β-pregnane). This product was present in large amounts in the late-pregnancy urine extracts and could be crystallized directly from the non-ketonic acetylated fraction (or after countercurrent distribution; see Expt. 3 below); it was further purified by chromatography as described above.

5β-Pregnane-3α:20α-diol demonstrated to be the major radioactive metabolite in the non-ketonic fraction

The procedure for estimating 5β-pregnane-3α:20α-diol was modified in Expt. 3 as follows.

The non-ketonic acetates (755·2 mg., 96% of original fraction) were dissolved in light petroleum–ether (2:1, v/v; 30 ml.), filtered through Al_2O_3 (6·0 g.) and eluted with the same solvent mixture, thereby removing a small amount of very dark pigment. The eluates furnished a red semi-crystalline mass (712·4 mg.) which was subjected to an 8-transfer countercurrent-distribution procedure with the aid of separating funnels; the solvent system was light petroleum–90% (v/v) methanol (50 ml.:156 ml.), which gave an effective partition coefficient (K) of 1·0 for 3α:20α-diacetoxy-5β-pregnane. [The solvents had been mutually saturated before use by shaking 1·0 vol. of light petroleum (b.p. below 40°) with 0·9 vol. of methanol and 0·1 vol. of water in a separating funnel.] The contents (both phases) of each funnel (0–8) were evaporated to dryness; the weight and specific activity of the residues are given in Table 3. The tritium determinations were made in a liquid scintillation counter. The contents of funnels 7 and 8 were highly pigmented and these determinations may have been in error by about $\pm 20\%$; the contents of the remaining funnels were colourless or only slightly pigmented. According to Table 3 radio-inert, amorphous material appeared at both ends of the countercurrent distribution, and the central funnel, 4, contained the largest amount of white crystalline material with the highest specific activity; the partitioning behaviour of the radioactivity approximated to that of 3α:20α-diacetoxy-5β-pregnane. Indeed, as is described below, this progesterone metabolite accounted for about 84% of the isotope content of the non-ketonic acetates; a lower figure of 72% (Table 1) was based on the isotope content of the original non-ketonic fraction which was determined by a combustion procedure. The crystalline residue from funnel 4 (96·9 mg., 92% of original fraction; 3·67 μc) gave after repeated crystallization 33·5 mg. of the diacetate, specific activity 0·0482 μc/mg. The mother liquors were estimated by isotope-dilution procedure to contain 43·3 mg. of the same compound. Hence a total of 76·8 mg. of the diacetate was present, accounting for 79% of the weight of the residue from funnel 4 and 101% of its isotope content. The 3α:20α-diacetoxy-5β-pregnane content of the total non-ketonic fraction was obtained by dividing the weight of diacetate in funnel 4 by the factor 0·273, since K equals 1·0. The countercurrent-distribution procedure and calculations have been described by Williamson & Craig (1947).

RESULTS

According to Table 1 31% (average value; range 16–56%) of the isotope administered as [16-^{3}H]-progesterone was recovered in the neutral urinary extracts of all subjects; the isotope was distributed between the ketonic and non-ketonic fractions in a ratio of about 1:4. The percentage recovery of tritium as urinary 5β-pregnane-3α:20α-diol was 6, 15 and 14% in the pregnant women, and 27 and 14% in the oophorectomized–hysterectomized subjects. According to Table 2 the endogenous production of progesterone ranged from 212 to 284 mg./day in advanced pregnancy.

Determination of the percentage recovery of tritium as 5β-pregnane-3α:20α-diol

From urine of oophorectomized–hysterectomized subjects. To a measured fraction, a, of the non-ketonic fraction a weighed amount, y, of inert 5β-pregnane-3α:20α-diol was added. The mixture was acetylated, chromatographed and repeatedly crystallized to afford 3α:20α-diacetoxy-5β-pregnane, of constant melting point, 164·5–165° (uncorrected; Kofler-type hot-stage apparatus). This product was

Table 1. *Recovery of tritium after administration of* [16-^{3}H]*progesterone in urine*

The figures in columns 5–7 refer to the amount recovered after acid hydrolysis and ether extraction, as described in detail in the text. The specific activity of the [16-^{3}H]progesterone was 22·2 μc/mg.

Exptl. subjects	Expt. no.	Amount of [16-^{3}H]-progesterone injected (μc)	(mg.)	Percentage of injected tritium recovered in urine: Neutral fraction (NK + K)	Non-ketonic fraction (NK)	Ketonic fraction (K)	Ratio $\left(\frac{K}{NK+K}\right)$	5β-Pregnane-3α:20α-diol
Oophorectomized–hysterectomized group	2	80·3	3·62	56	47	8·6	0·15	27
	5	102·0	4·60	24·5	20	4·5	0·18	14
Pregnant group								
25th week	1	185·0	8·31	16	10	5·5	0·34	5·7
36th week (twins)	3	106·0	4·77	36	22	14·0	0·39	15·0
34th week	4	103·0	4·65	22	17	5·0	0·23	14·0

Table 2. *Calculated daily endogenous production of progesterone and daily urinary 5β-pregnane-3α:20α-diol excretion in advanced pregnancy*

For method of calculation see text.

Stage of pregnancy (week)	Expt.	Urinary 5β-pregnane-3α:20α-diol excretion (mg./day)	Endogenous progesterone production (mg./day)
25	1	16	275
36 (twins)	3	42	284
34	4	30	212

Table 3. *Countercurrent distribution of acetylated non-ketonic fraction (713 mg.) from Expt.* 3

Solvent system: light petroleum (b.p. below 40°)–90% (v/v) methanol (50 ml.:156 ml.), giving an effective partition coefficient, K, of 1·0 for 3α:20α-diacetoxy-5β-pregnane; 8 transfers.

Funnel no.	Wt. of residue (mg.)	Specific activity (μc/mg.)	Total isotope content (μc)
0	105 (oil)	0·0049	0·52
1	41 (oil)	0·029	1·20
2	49 (semi-cryst.)	0·038	1·85
3	77 (cryst.)	0·036	2·80
4	105 (cryst.)	0·038	3·99
5	105 (cryst.)	0·036	3·73
6	85 (semi-cryst.)	0·026	2·19
7	72 (oil)	0·013	0·94
8	61 (oil)	0·0013	0·08

not in all instances examined for radiochemical purity; in Expt. 1, the specific activity of the isolated 3α:20α-diacetoxy-5β-pregnane remained unchanged on recrystallization. Possible contamination with ketonic radiometabolites appeared minimal in view of the rigorous fractionation of the neutral extract into ketonic and non-ketonic portions. Possible contamination with non-ketonic metabolites such as 3α:20α-diacetoxy-5α-pregnane was considered, but this also seemed negligible: analysis of the non-ketonic fraction (Expt. 2) by isotope dilution indicated that the respective 5β- and 5α-pregnanediols were present in a ratio of about 19:1. Kyle & Marrian (1951), employing conventional analytical methods, reported similarly a ratio of about 30:1 in a sample of human pregnancy urine. That 5β-pregnane-3α:20α-diol is the predominant non-ketonic radiometabolite was demonstrated in Expt. 3 by countercurrent-distribution procedures (see above).

The specific activity, C', of the isolated 3α:20α-diacetoxy-5β-pregnane was determined and the tritium content, z, of the free diol in the total non-ketonic fraction was obtained from the equation $z = (1{\cdot}26C')\, y/a$. The correction factor, 1·26, is the ratio of the molecular weights of the diacetate and the free diol (404·5 and 320·4 respectively). The percentage recovery of tritium as the urinary diol was readily obtained, the amount of isotope administered being known (Table 1). Undiluted urinary 5β-pregnane-3α:20α-diol was not isolated because the amount was too small, endogenous progesterone production being minimal in these subjects; since the amount by weight, x, of urinary diol in a was negligible by comparison with y, it was omitted from the calculations.

From pregnancy urine. The amount of 5β-pregnane-3α:20α-diol in the pregnancy-urine extracts being considerable, it was easily isolated as the diacetate from the acetylated non-ketonic fraction and its specific activity, C, determined. To a measured portion, a, of the acetylated non-ketonic fraction, a weighed amount, y, of inert 3α:20α-diacetoxy-5β-pregnane was added; the diacetate was isolated again and its specific activity, C', determined. The amount x of the diacetate in the entire non-ketonic fraction was obtained from the equation $x = yC'/a(C - C')$. The tritium content z of the total urinary 3α:20α-diacetoxy-5β-pregnane was obtained from

the equation $z = xC$. The daily urinary 5β-pregnane-3α:20α-diol excretion (Table 2) was obtained by dividing x by the number of days of the urine-collection period.

Estimation of the endogenous progesterone production in pregnancy

One need only determine the specific activity C of the undiluted urinary 3α:20α-diacetoxy-5β-pregnane in order to calculate the daily endogenous progesterone production P, since the amount by weight w of the [16-^{3}H]progesterone injected and its specific activity S were known. The equation employed was $P = 0{\cdot}98Sw/Cd$, where d is the number of days of the urine collection period; the correction factor, 0·98, is the ratio of the molecular weights of progesterone and 5β-pregnane-3α:20α-diol (314·4 and 320·4 respectively); the other symbols have been defined above. The same value for P would, of course, be obtained by dividing the daily urinary 5β-pregnane-3α:20α-diol excretion (Table 2) by the percentage recovery of tritium as the urinary diol (Table 1) and multiplying by the correction factor 0·98.

DISCUSSION

There is no support from the limited data (Table 1) in this study for the view (Venning & Browne, 1940; Sommerville & Marrian, 1950; Guterman, 1953) that the conversion of progesterone into pregnanediol is greatly increased (as much as threefold) in pregnancy as compared with that which obtains in the non-pregnant state in the absence of luteal function. Nor could such support be obtained in a recent study by Klopper & Michie (1956), who administered non-isotopic progesterone to three patients in early pregnancy: these workers stated that the analytical method they employed was new, precise, accurate and specific for pregnanediol, and that it was possible to estimate the additional amount in pregnancy urine in a way not available to the early workers. Indeed, from recent investigations (Marrian *et al.* 1954; Klopper & Michie, 1956) it appears that the course of progesterone metabolism with respect to pregnanediol formation is influenced neither by the uterus or the corpus luteum nor by the administration of oestrogen. Similarly the metabolism of oestrone in advanced pregnancy has been shown with the aid of deuterium-labelling (Pearlman, Pearlman & Rakoff, 1954) not to depart significantly from that in non-pregnancy. In view of the quantities of both progesterone and oestrogen produced in late pregnancy, it is noteworthy that these hormones do not appreciably affect each other in their metabolism, although they markedly modify their respective physiological activities (Courrier, 1950). The earlier observations that the metabolism of oestrogen and progesterone is influenced by the endocrine status of the organism, and hence is somehow linked with their physiological utilization, thus cannot be confirmed. In advanced pregnancy the body appears to be flooded with steroid sex hormones in a process which is wasteful in that they are rapidly metabolized into biologically inactive products in organs not associated with the reproductive process, notably the liver; these metabolic transformations overshadow any that might occur in the uterus or mammary glands.

The endogenous production of progesterone in advanced pregnancy, as calculated by the isotope-dilution method, is about 0·25 g./day (Table 2). It may be much higher in some women in view of the wide range among individuals in urinary pregnanediol excretion rates (Venning, 1948) and the varying degree of hormone metabolism. One wonders how much of this progesterone, which in the human species arises chiefly from the placenta with advancing pregnancy, undergoes transformation into corticoids in the maternal or foetal adrenal glands; the hormonal inter-relationships between these organs have recently been reviewed by Davis & Plotz (1956).

The question may be raised as to the validity of using [16-^{3}H]progesterone in the study of the intermediary metabolism of the hormone not involving loss of the C-17 side chain. It has been assumed in this study that no loss of tritium occurred in the *in vivo* conversion of progesterone into 5β-pregnane-3α:20α-diol. To establish this point a solution of [16-^{3}H]progesterone and [4-^{14}C]progesterone was injected into a cancer patient and 5β-pregnane-3α:20α-diol was subsequently isolated from the urine (unpublished experiments in collaboration with Dr J. F. Tait and others); the ^{3}H:^{14}C ratios of the urinary diol and of the hormone injected were identical within experimental error when determined by the combustion–gas-counting procedure. The recovery of radioactivity in the urine after [16-^{3}H]progesterone administration (Table 1) averaged 31% (range 16–56%), which is comparable with that obtained after the administration of [4-^{14}C]progesterone to human subjects (Bradlow & Gallagher, 1955; Wiest, Fujimoto & Sandberg, 1955); this agrees also with the finding that 5β-pregnane-3α:20α-diol is the major radioactive metabolite. Davis, Plotz, Le Roy, Gould & Werbin (1956) injected [4-^{14}C]progesterone into a pregnant patient and recovered 25% of the administered radioactivity from the urine and 28·5% from the faeces. (It is likely, in view of the latter observation, that the faeces in the present study contained tritium, but this was not ascertained.) Other examples of the effective application of hydrogen-isotope labelling in investigations of steroid-hormone metabolism may be mentioned, e.g. the conver-

sion of cholesterol into pregnanediol (Bloch, 1945), oestrone metabolism (Pearlman *et al.* 1954) and testosterone and cortisone metabolism (Gallagher *et al.* 1954). There are certain biochemical limitations in the valid use of deuterium or tritium, and broad aspects of the problem are discussed by Kamen (1951).

SUMMARY

1. [16-^{3}H]Progesterone was injected intramuscularly into three women in the last trimester of pregnancy and into two oophorectomized–hysterectomized patients who served as a control group. The urinary recovery of tritium in the neutral ketonic and non-ketonic fractions and as 5β-pregnane-3α:20α-diol was determined.

2. Of the radioactivity injected 31% (average value for all subjects) was recovered in the neutral urinary extracts; the isotope was distributed between the ketonic and non-ketonic fraction in a proportion of about 1:4. 5β-Pregnane-3α:20α-diol was the major radioactive metabolite.

3. The percentage recovery of progesterone as urinary 5β-pregnane-3α:20α-diol was calculated by isotope-dilution procedures to be 6, 15 and 14% in the pregnant subjects and 27 and 14% in the oophorectomized–hysterectomized subjects. These findings do not support earlier observations (Venning & Browne, 1940; Sommerville & Marrian, 1950; Guterman, 1953) by conventional analytical methods that the percentage recovery of the metabolite in pregnancy is increased about threefold.

4. The endogenous production of progesterone was similarly calculated to be of the order of 0·25 g./day in late pregnancy.

5. The validity of using [16-^{3}H]progesterone in the study of the intermediary metabolism of the hormone is discussed.

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The Metabolism of Thyroid Hormones

2. DETECTION OF THYROXINE AND TRI-IODOTHYRONINE IN HUMAN PLASMA*

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The introduction of paper chromatography and the availability of radio-iodide enabled Laidlaw (1949) to show that the circulating thyroid hormone was thyroxine and not thyroglobulin or a peptide, a conclusion supported by the work of Taurog, Chaikoff & Tong (1950) and Rosenberg (1951). After the discovery of tri-iodothyronine (Gross & Pitt-Rivers, 1952*a*, *b*, 1953*a*, *b*; Roche, Lissitzky & Michel, 1952*a*, *b*) it became clear that many of the earlier methods would not have been capable of differentiating between thyroxine and the new substance. The paper-chromatographic techniques

* Part 1: Sprott & Maclagan (1955).

previously used employed for the most part solvents which would not separate the two compounds. The use of new solvent systems, however, has enabled several groups of workers to detect the presence of both compounds in the plasma after the administration of radio-iodide (Dingledine, Pitt-Rivers & Stanbury, 1955; Benua & Dobyns, 1955).

In view of the possibility of radiation artifacts it appeared desirable to conduct parallel studies by non-radioactive methods. The introduction of ceric sulphate–arsenious acid as an extremely sensitive reagent for the detection of thyroxine and related compounds on paper chromatograms has offered an alternative approach (Bowden & Maclagan, 1954; Bowden, Maclagan & Wilkinson, 1955; Maclagan, Bowden & Wilkinson, 1955). The present paper describes the application of this reagent to the study of the iodine-containing compounds of plasma without using radioactive materials. A preliminary account of this work has already appeared (Wilkinson & Maclagan, 1955).

MATERIALS AND METHODS

All glassware was cleaned with chromic acid and immediately before use was rinsed with 0·1 N-NaOH and iodine-free water. Water distilled in a glass-lined still proved satisfactory when tested with ceric sulphate and arsenious acid (Bowden *et al.* 1955). 'Deionized' water having a specific resistance greater than 0·8 MΩ/cm., obtained from an Elgastat Deionizer, was used in the preparation of all solutions.

Paper chromatography. Whatman no. 1 paper was used. Chromatograms were developed by the ascending technique with one or more of the following solvent systems: butanol–dioxan–NH_3 (Gross, Leblond, Franklin & Quastel, 1950); *iso*pentanol–NH_3 (Roche, Deltour, Michel & Velez, 1953); butanol–2 N-acetic acid; or 2-methylbutan-2-ol–NH_3 (Gleason, 1955). The system containing dioxan was renewed every 2 weeks to avoid complications due to peroxide formation (Taurog, Briggs & Chaikoff, 1952; Owen, McKenzie & Orvis, 1956). After the chromatograms had been dried in an iodine-free atmosphere at room temperature, the positions of the iodine-containing compounds were located by the ceric sulphate–arsenious acid reagent (Bowden *et al.* 1955).

Thyroxine butyl ester. Thyroxine (50 mg.) was heated under reflux with 80% (v/v) aqueous butanol (20 ml.) and 10 N-HCl (0·2 ml.) for 1 hr., after which the solvent was removed under reduced pressure. The residue was suspended in 0·05 N-Na_2CO_3 (3 ml.) and continuously extracted with ether. Evaporation of the solvent, followed by recrystallization of the residue from ethyl acetate, gave thyroxine butyl ester, 20 mg. m.p. 89° (decomp.) (Found: C, 27·8; H, 2·7; N, 1·8; I, 60·0. $C_{19}H_{19}O_4NI_4$ requires C, 27·4; H, 2·3; N, 1·7; I, 60·9%). It gave a negative ninhydrin test in solution, though on paper sprayed with ninhydrin reagent it produced a pink spot. When sprayed with diazotized sulphanilic acid solution a spot on paper gave a red colour, indicating the presence of a free hydroxyl group. The same product was obtained when 10% (v/v) aqueous butanol was used as solvent.

3:5-Di-iodo-L-tyrosine butyl ester. 3:5-Di-iodo-L-tyrosine (2 g.) was heated under reflux with butanol (90 ml.) and 10 N-HCl (10 ml.). The residue obtained by evaporation under reduced pressure was suspended in 0·05 N-Na_2CO_3 (10 ml.) and continuously extracted with ether. The ether-insoluble material consisted of unchanged di-iodotyrosine. The ether was evaporated and the residue crystallized from ethanol (charcoal). The *ester* (1·0 g.) had m.p. 135° (Found: C, 32·3; H, 3·8; N, 2·7; I, 52·5. $C_{13}H_{17}O_3NI_2$ requires C, 31·9; H, 3·5; N, 2·9; I, 51·8%). It gave a positive diazotized sulphanilic acid test and a negative ninhydrin test.

By similar means thyroxine was converted into its methyl and ethyl esters and tri-iodothyronine into its butyl ester. These substances were not isolated but their presence was demonstrated chromatographically. Hydrolysis with NaOH regenerated the parent amino acids. The R_F values of the esters are listed in Table 1.

Typical procedure for the extraction of thyroxine and tri-iodothyronine from plasma

Blood was collected by venepuncture and the plasma separated within 1–2 hr.; oxalate or heparin was used as anticoagulant. The plasma (10 ml.) was shaken with methanol (24 ml.) for 1–2 min. (Brown & Jackson, 1954). The protein was removed by centrifuging and the supernatant was separated. The protein precipitate was washed twice with 70% (v/v) aqueous methanol (10 ml.). The combined washings and supernatant were evaporated to

Table 1. *R_F values of esters of di-iodotyrosine and iodothyronines*

Ascending method on Whatman no. 1 paper. For references to composition of solvent mixtures see Methods.

Amino acids	Esters	R_F values in: Butanol–dioxan–2 N-NH_3	2-Methylbutan-2-ol–2 N-NH_3	*iso*Pentanol–2 N-NH_3
3:5-Di-iodotyrosine	—	0·18	0·02	0·02
3:5-Di-iodotyrosine	Butyl	0·86	0·75	0·83
3:5-Di-iodothyronine	—	0·72	0·58	0·50
3:5-Di-iodothyronine	Butyl	0·97	0·93	0·95
3:5:3′-Tri-iodothyronine	—	0·70	0·54	0·39
3:5:3′-Tri-iodothyronine	Butyl	0·95	0·89	0·89
Thyroxine	—	0·59	0·32	0·24
Thyroxine	Methyl	0·86	0·84	0·87
Thyroxine	Ethyl	0·91	0·87	0·94
Thyroxine	Butyl	0·90	0·89	0·85

dryness at 20±5° at 2–3 mm. The residue was incubated with 0·06 M-phosphate buffer of pH 7·4 (2 ml.) and a solution of crystalline trypsin (0·2 mg.) in 0·05 N-HCl (0·2 ml.) at 37° for at least 2 hr., or overnight if convenient. Methanol (4 ml.) and light petroleum (b.p. 40–60°) (6 ml.) were added; the mixture was shaken vigorously and centrifuged and the upper layer was discarded. The flask was rinsed with light petroleum (6 ml.), which was then used to re-extract the lower layer in the centrifuge tube. This process was repeated twice until the aqueous methanolic layer had been extracted at least four times. The lower layer was then returned to the flask, the small amount of solid being rinsed with 70% (v/v) methanol and discarded. The combined methanolic layer was evaporated at 20±5° at 2–3 mm. The residue was dissolved in 0·02 N-HCl (3 ml.) (the pH of the resulting solution was about 5) and extracted three times with butanol (3 ml., 2 ml., 2 ml.) below 15°: separation of the layers was completed by centrifuging. The combined extract was washed with water (6 ml.) and evaporated at 15±5° at 2–3 mm. as rapidly as possible. The gummy residue (about 0·2 mg.) was dissolved in 0·02 N-Na_2CO_3 (2 ml.) and the solution twice extracted with ether (6 ml.). The aqueous layer was adjusted to pH 4–5 with 0·02 N-HCl (2 ml.) and extracted as above with butanol. The extract was washed with water and evaporated as described above. The residue was dissolved in 50 μl. of butanol and 10 μl. portions (equivalent to 2 ml. of plasma) were chromatographed, except in certain cases of hypothyroidism when larger amounts of the extracts (up to 33 μl.) were used. Thyroxine and tri-iodothyronine markers (0·05–0·2 μg.) were always run alongside the extracts. Stained spots were assessed visually within 1 hr. (Table 2).

Determination of thyroxine and tri-iodothyronine on paper chromatograms by elution method

Rectangles of paper containing the spots of thyroxine and tri-iodothyronine were cut from the dried unstained paper after location by a stained marker strip. Each was folded and immersed in water (3 ml.) and 0·2 N-arsenious acid (Leffler, 1954) (2 ml.) at 25° for 20 min. At 30 sec. intervals 0·02 N-ceric sulphate (Leffler, 1954) (1 ml.) was added to each tube, which was then shaken for 1 hr. at 25°. Each solution was then decanted from the paper and the percentage transmission measured at 30 sec. intervals in an EEL absorptiometer (Ilford 601 violet filter) adjusted for 100% transmission with water. Calibration curves are shown in Fig. 1.

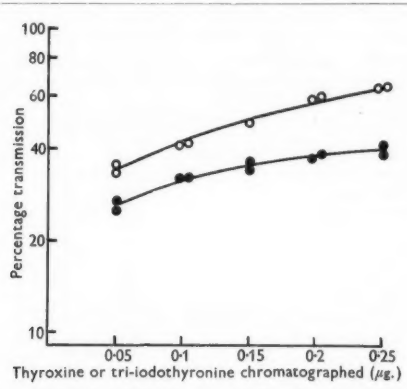

Fig. 1. Typical calibration curves for the determination of thyroxine (○) and tri-iodothyronine (●) by elution from paper chromatograms with the ceric sulphate–arsenious acid reagent.

RESULTS

Stability of solutions of thyroxine and tri-iodothyronine

In aqueous 0·02 N-sodium carbonate at room temperature thyroxine (1 mg./10 ml.) yielded in 2–3 days iodide and a substance which on paper chromatography behaves like tri-iodothyronine (Wilkinson, Sprott, Bowden & Maclagan, 1954). In 50% (v/v) aqueous propylene glycol adjusted to pH 8–9 with diethanolamine, thyroxine underwent similar decomposition more slowly; so that iodide was detected after about 10 days. A 0·0025 N-solution of NaOH in 99% aqueous butanol also caused deiodination. At higher temperatures, the same type of decomposition occurred more rapidly. Tri-iodothyronine behaved similarly in alkaline solution, and after several months iodide only was detectable. In acid alcohols, however, esterification took place. Fast-moving spots (R_g 0·90–0·95) were observed when solutions of either substance in 0·1 N-HCl in 99% butanol were stored at 20°+5° for prolonged periods and chromatographed in butanol–dioxan–ammonia. This process eventually went to completion. When the solutions were heated at 100°, free thyroxine or tri-iodothyronine could not be detected after 1 hr. The products were proved to be the butyl esters by comparison of R_g values with authentic specimens. On treatment with 0·05 N-NaOH at 100° for 10 min. the esters yielded thyroxine and tri-iodothyronine respectively. Di-iodotyrosine was also converted into its butyl ester in acid butanol, and other alcohols gave corresponding esters with each of the iodinated amino acids studied. It is clear, therefore, that on storage in aqueous butanol containing hydrochloric acid esterification is liable to occur. The proportions of water and butanol did not seem to have any effect on the reaction, for esterification occurred almost as readily in a solution of butanol in water as in water-saturated butanol.

To exclude esterification as a source of artifacts the pH was kept above 4 during all stages of the extraction. Under these conditions no evidence of esterification was detected.

Recovery of added thyroxine

The extract prepared from 5 ml. of plasma to which 0·1 μg. of thyroxine and 0·1 μg. of tri-iodothyronine had been added gave satisfactory chromatograms. The sizes and positions of the spots

produced after treatment with the ceric sulphate–arsenious acid reagent were comparable with those obtained from marker solutions containing similar quantities of thyroxine and tri-iodothyronine.

The quantitative recovery of added thyroxine was measured in two stages: (1) from plasma into the final butanol extract; (2) from the butanol extract to the developed chromatogram and colorimetric determination. The recovery during the first stage was determined by adding 0·2–0·4 μg. of ^{131}I-labelled thyroxine to plasma and comparing the counting rates of the plasma and the final extracts. After allowance was made for radioactive decay, the recoveries in the five experiments were 90, 90, 97, 91 and 87 %.

During the second stage thyroxine and tri-iodothyronine (0·1 or 0·2 μg.) were added to extracts equivalent to 2 ml. of plasma which were chromatographed, and the iodine compounds were determined by the elution method. Duplicate results indicated good reproducibility and the recoveries of added material ranged from 70 to 110 % (mean 87 ± 10 %, 16 expts.) for thyroxine and from 50 to 90 % (mean 71 ± 11 %, 16 expts.) for tri-iodothyronine.

Clinical results by visual assessment

The results obtained during the examination of the plasma of a number of individuals in various states of thyroidal activity are summarized in Table 2. The euthyroid group includes some

Table 2. *Plasma thyronines determined by visual assessment method*

0 = Not detected; + = detectable with difficulty or present in small amount; + + = easily detectable; + + + = easily detectable in relatively large amounts.

Subject					
No.	Sex	Age	State	Thyroxine	Tri-iodothyronine
1	F.	18	Thyrotoxic	+ + +	+ +
2	F.	37	Thyrotoxic	+ +	+ +
3	F.	39	Thyrotoxic	+	+ + +
4	F.	70	Thyrotoxic	+ +	+
5	F.	50	Thyrotoxic	+	+
6	F.	52	Thyrotoxic	+	+ + +
7	M.	23	Thyrotoxic	+	+ +
8	F.	37	Thyrotoxic	+ +	+ + +
9	F.	35	Thyrotoxic	+	+ +
10	F.	42	Thyrotoxic	+ +	+
11	F.	63	Thyrotoxic	+ + +	+
12	F.	50	Thyrotoxic	+ +	+
13	M.	42	Thyrotoxic	+ + +	+
14	F.	58	Thyrotoxic	+ +	+ + +
15	M.	52	Thyrotoxic	+ +	+ + +
16	F.	32	Thyrotoxic	+ +	+
17	F.	36	Hyperthyroid	+ + +	+
18	F.	24	Euthyroid	+	+
19	F.	20	Euthyroid	+	+
20	F.	30	Euthyroid	+	+
21	F.	61	Euthyroid	+	+ +
22	M.	44	Euthyroid	0	+ +
23	M.	33	Euthyroid	+	+ +
24	F.	32	Euthyroid	+ +	0
25	F.	8	Euthyroid	+ +	+
26	F.	21	Euthyroid	+ +	+
27	F.	56	Hypophysectomy regeneration	+ +	+
28	F.	64	Euthyroid (non-toxic goitre)	+	0
29	M.	14	Euthyroid (non-toxic goitre)	+	+
30	F.	34	Hypothyroid	+ +	0
31	F.	2	Hypothyroid	0	0
32	F.	53	Hypothyroid	+	0
33	M.	42	Hypothyroid	+	0
34	F.	62	Myxoedema	0	0
35	—	—	Myxoedema	0	0
36	F.	65	Myxoedema	0	0
37	F.	54	Myxoedema	0	0
38	M.	42	Myxoedema	0	0
39	—	—	Myxoedema	0	0
40	F.	67	Myxoedema	+	0
41	F.	67	Myxoedema	0	0
42	F.	57	Hypothyroid	+ +	0
43	F.	28	Hypothyroid	+ +	0

members of the laboratory staff who were in good health, as well as hospital patients under investigation for conditions not involving the thyroid gland. Tri-iodothyronine was detected in the plasma of all members of the thyrotoxic group and nearly all the normal controls, but was not found in any of the 14 hypothyroid subjects.

The possibility that thyroxine was partly converted into the tri-iodo compound during the extraction procedure was eliminated as follows: (*a*) radiothyroxine and carrier tri-iodothyronine were added to normal plasma, which was then extracted and the extracts were chromatographed. Radioautographs of the chromatogram showed single thyroxine spots: no labelled tri-iodothyronine was detected. (*b*) A large excess (0·4 μg.) of non-radioactive thyroxine was added to the plasma of a hypothyroid patient previously shown not to contain tri-iodothyronine. The chromatogram showed only thyroxine.

In addition to the demonstration that the spots on the chromatograms were coincident with similar spots containing added thyroxine and tri-iodothyronine, identification was confirmed by developing the chromatograms in a number of different solvent systems. No known compound has the same R_F as thyroxine in each of the systems used, but 3:5-di-iodo-4-(4-hydroxy-3:5-di-iodophenoxy)-phenylacetic acid has very similar chromatographic properties to tri-iodothyronine. As the presence of this substance has been demonstrated in biological systems (Roche, Michel & Jouan, 1957; Albright, Larson, Tomita & Lardy, 1956) it was desirable to distinguish between the two compounds. At the suggestion of Professor S. Lissitzky the behaviour of the two compounds on paper ionophoresis was examined. In barbitone buffer of pH 8·6 at a potential of 150 v tri-iodothyronine did not move in 16 hr., whereas 3:5-di-iodo-4-(4-hydroxy-3:5-di-iodophenoxy)phenylacetic acid moved 4·6 cm. towards the anode in 4 hr. Under similar conditions thyroxine moved about 0·2 cm. in 16 hr. and 3:5-di-iodo-4-(4-hydroxy-3-iodophenoxy)phenylacetic acid 2·8 cm. in 4 hr. in the same direction. Extracts of plasma of two cases of thyrotoxicosis were chromatographed in 2-methylbutan-2-ol–ammonia for 16 hr.; the papers were dried and placed in an electrophoresis tank such that the direction of the electric potential was perpendicular to the chromatographic flow. After 4 hr. at 150 v the spots of R_F 0·54 had not moved in response to the electric potential and consequently were those of tri-iodothyronine.

Several plasma extracts contained an unidentified brown pigment (R_F in 2-methylbutan-2-ol–NH_3, 0·68) which, although easily distinguished from tri-iodothyronine (R_F 0·54) in this solvent, overlapped this region in others. This substance fluoresced yellow–brown in ultraviolet light and interfered with the preparation of permanent records by ultraviolet photography (Bowden *et al.* 1955). In marked contrast with the behaviour of the iodothyronines and iodotyrosines it decolorized the ceric sulphate–arsenious acid reagent only after 12–24 hr.

In one of the hypothyroid patients and in two of the normal individuals small amounts of an unknown iodine-containing component were detected. It had an R_F value of 0·54–0·58 in *iso*pentanol–NH_3 and 0·60 in 2-methylbutan-2-ol–NH_3. Unfortunately there was not sufficient material for further investigation.

Clinical results by quantitative elution method

The results obtained from 10 patients by this procedure are shown in Table 3. Although this small series is an inadequate test of the method it will be seen that euthyroid patients have plasma thyroxine values up to 5 μg./100 ml. and plasma tri-iodothyronine values up to 0·5 μg./100 ml. The high values obtained in the first 5 cases were expected. In addition the distinction between

Table 3. *Plasma thyronines determined by elution method*

Subject			State	Thyroxine (μg./100 ml.)	Tri-iodothyronine (μg./100 ml.)
No.	Sex	Age			
12	F.	50	Thyrotoxic	5	1
13	M.	42	Thyrotoxic	10	<1
15	F.	52	Thyrotoxic	5	4
43	F.	28	Hypothyroid (after treatment with tri-iodothyronine)	2·5	9
44	M.	45	Patient had taken thyroid extract	32	4·5
44	M.	45	After 6 weeks in hospital without thyroid medication	2·5	<0·5
45	F.	33	Hypothyroid	1·5	<0·5
46	M.	21	Carcinoma of thyroid	4·5	<0·5
47	M.	32	Euthyroid	4·5	0·5
48	F.	31	Euthyroid	5	0·5
49	F.	44	Hypothyroid	2·5	<0·5

case 43 (tri-iodothyronine therapy) and case 44 (treated with thyroid extract) is clearly shown. The procedure was too time-consuming for routine use, but it may have a place in special investigations.

DISCUSSION

The results summarized in Table 2 show that tri-iodothyronine is a true component of the organic iodine fraction of normal and thyrotoxic plasma and that it is somewhat more easily detectable in the latter than in the former. This is in complete agreement with earlier work with radio-iodine (Dingledine *et al.* 1955; Benua & Dobyns, 1955). The latter authors suggested that the amount of circulating tri-iodothyronine was probably related to the degree of thyrotoxicosis. The possibility that the tri-iodothyronine detected by these workers was a radiation artifact could not entirely be discounted, though Benua, Dobyns & Ninmer (1955), who found it as early as 1 hr. after the administration of radio-iodide, suggest that radiation damage is unlikely to be responsible. The present work confirms the conclusion drawn from the radioactive investigations, since there can be no question of interference with the gland.

Tri-iodothyronine was uniformly absent in the fourteen hypothyroid patients, though thyroxine was found in six of them. Eight of the patients in this group suffered from myxoedema and in one of these thyroxine was detected in the plasma. In this subject it is possible that the myxoedema may have arisen as a result of a peripheral failure to convert thyroxine into tri-iodothyronine as reported by Kurland, Hamolsky & Freedberg (1955).

The quantitative results shown in Table 3 suggest that the method could be used to demonstrate variations in the proportions of thyroxine and tri-iodothyronine in the plasma. Such variations may account for discrepancies which have occasionally been observed between the plasma protein-bound iodine levels and the thyroid status of the individual (Mowbray & Tickner, 1952; Schultz, Sandhaus, Demorest & Zieve, 1954; Vannotti, 1957).

SUMMARY

1. A method for extracting the iodine-containing components of plasma has been described. Paper chromatography combined with paper ionophoresis has enabled the circulating substances to be identified as thyroxine and tri-iodothyronine.

2. Thyroxine and tri-iodothyronine have been detected in the plasma of sixteen thyrotoxic patients and fifteen normal or euthyroid subjects, but tri-iodothyronine was not found in the plasma of any of the seventeen hypothyroid patients.

3. The stability of solutions of thyroxine and tri-iodothyronine in a number of solvent systems has been studied. In aqueous alkaline solution both substances slowly liberated iodide, but in acid alcoholic solutions both compounds were rapidly esterified even in the presence of relatively large concentrations of water.

4. A method devised for the determination of thyroxine and tri-iodothyronine on paper chromatograms has been used to estimate the circulating levels of these substances in ten patients.

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The Determination of Ketone Bodies

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In the determination of ketone bodies it is customary to convert acetoacetic acid and β-hydroxybutyric acid into acetone for estimation. This may be brought about during distillation (Shaffer, 1908–09; Shipley & Long, 1938; Weichselbaum & Somogyi, 1941) or by refluxing or heating in pressure bottles (Van Slyke, 1917; Greenberg & Lester, 1944; Michaels, Margen, Liebert & Kinsell, 1951). When distillation is employed preformed acetone plus acetoacetic acid may be estimated separately from β-hydroxybutyric acid in the one blood or urine filtrate; this is not possible when refluxing or pressure bottles are used. However, the latter methods permit greater reproducibility in the estimation of β-hydroxybutyric acid, since the acid-dichromate reagent used for the oxidation is maintained at constant concentration.

The present method combines the advantages of both procedures by using a combined reflux and distillation apparatus. Acetone in the distillates is converted into the 2:4-dinitrophenylhydrazone, which is extracted with carbon tetrachloride and estimated colorimetrically by a modification of the method of Greenberg & Lester (1944).

MATERIALS AND METHODS

Reagents. Sulphuric acid, 20N; potassium dichromate, 2·5% (w/v); freshly filtered 2:4-dinitrophenylhydrazine, 0·1% (w/v) in 4N-HCl; carbon tetrachloride; NaOH, 0·5N; barium hydroxide, approx. 0·3N; zinc sulphate, approx. 5% (w/v) $ZnSO_4,7H_2O$ (10 ml. of the barium hydroxide solution added drop by drop with phenolphthalein as indicator, should neutralize exactly 10 ml. of the $ZnSO_4$ solution). Distilled water is used throughout the determination. All reagents are of A.R. grade.

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Preparation of standard solutions

Acetone. A.R. acetone was redistilled three times, only the middle fraction boiling at 56·5° being retained. Standard solutions were prepared by weighing up to 1 g. of acetone in a stoppered tube and dissolving it in water. The possibility of loss of acetone due to volatilization was overcome by suspending the stoppered tube beneath water in a 2 l. volumetric flask and removing the stopper by means of an attached thread. This solution was made up to 2 l. and diluted as required.

Acetoacetic acid. Widmark (1920–21) found that acetoacetic acid in acid solution underwent complete decomposition to acetone, the determination of which could be used as a means of estimating quantitatively the acetoacetic acid. Accordingly, a solution of sodium acetoacetate, prepared by the method of Ljunggren (1924), was standardized with the present method.

β-Hydroxybutyric acid. The calcium–zinc salt of β-hydroxybutyric acid was prepared according to Shaffer & Marriott (1913–14) and Blunden (1938). Colourless needles and plates were obtained (m.p. 247·5–248·5° uncorr. with

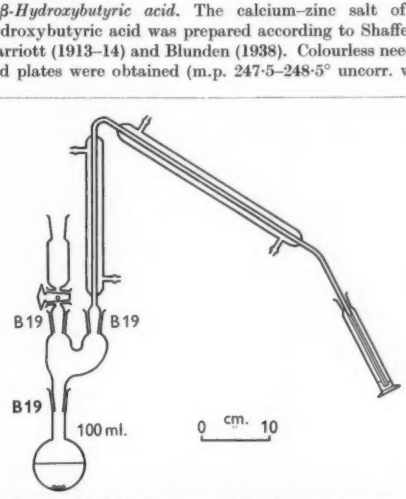

Fig. 1. Combined reflux and distillation apparatus.

decomp.). Sodium β-hydroxybutyrate (British Drug Houses Ltd.), which the manufacturers claim to be 98% pure (Thin & Robertson, 1952), was used also. Solutions were made from both preparations for use as standards.

Apparatus. An apparatus constructed from B19 standard ground-glass joints is employed (Fig. 1). The distillation tube (bore 8 mm.) is fused to a length of capillary tubing (bore 2 mm.) to form a delivery tube.

Procedure for the estimation of acetone plus acetoacetic acid and of β-hydroxybutyric acid

Deproteinization. Blood (0·1 ml.) is haemolysed by mixing it with water (7 ml.) in a centrifuge tube (15 ml.). The tube is stoppered and shaken. Barium hydroxide solution (0·4 ml.) is added and the tube shaken to ensure complete mixing, followed by the addition of $ZnSO_4$ solution (0·4 ml.) and further vigorous shaking. The mixture is centrifuged and the supernatant liquid filtered through Whatman no. 44 filter paper. The filtrate is used for the estimation of ketone bodies.

Suitably diluted urine (7 ml.) may be treated with the deproteinizing reagents in the same way as blood.

Distillation. The apparatus should be assembled with silicone high-melting-point grease between the joints. It is advisable to boil it out with water after assembly and before use each day. Into a 100 ml. flask, containing three small glass beads, is placed H_2SO_4 (8 ml.) and water (50 ml.), followed by 5 ml. of deproteinized filtrate. A 5 ml. hypodermic syringe, fitted with a stop and calibrated to deliver within ±0·1% may be used with advantage. This arrangement makes it possible to direct the filtrate into the reagents speedily and without loss on the sides of the flask. The flask is immediately fitted to the apparatus with cold water circulating through both condensers, and then boiled gently under reflux. After refluxing for 5 min., the circulating water is drained from the reflux condenser, allowing the vapour to distil and collect in a 25 ml. stoppered-type graduated cylinder containing 1 ml. of 2:4-dinitrophenylhydrazine reagent. The rate of distillation is increased progressively and the graduated cylinder should be clamped so that the delivery tube dips beneath the surface of the reagent. When 5 ml. of distillate has collected, the graduated cylinder is lowered slightly to expose the delivery tube, which is washed externally with a fine jet of water (4 ml.), and internally by allowing a further 5 ml. of distillate to collect in the cylinder. Immediately, distillation is stopped by circulating cold water through the reflux condenser. Potassium dichromate solution (2 ml.) is introduced into the apparatus via the tap funnel, followed by water (10 ml.), which should rinse into the apparatus any dichromate remaining in the funnel. After refluxing for 40 min. a distillate is collected as described above. Boiling must be continuous throughout the procedure to avoid sucking back laboratory air. The first distillate is analysed for its acetone content and represents acetone plus acetoacetic acid. The second distillate contains the acetone derived from the oxidation of β-hydroxybutyric acid.

Colorimetric estimation of acetone. The distillate which has collected in the 25 ml. graduated cylinder is made up to exactly 15 ml. with water. Carbon tetrachloride (6 ml.) is added from an automatic burette and the cylinder is stoppered and shaken mechanically for 150 min. After it has separated, the supernatant acid solution is sucked off. Without shaking it, the cylinder is filled twice with water, which is sucked off each time; finally, NaOH solution (9 ml.) is added. The solutions are shaken for a further 5 min. and allowed to stand. When the carbon tetrachloride layer is perfectly clear the optical density is read in a spectrophotometer at 350 or at 410 mμ, depending on the concentration of acetone present. With the Spekker absorptiometer a violet filter (Ilford 600 or 601) should be used. Blank determinations are carried out with water in place of blood or urine. A calibration curve is obtained by similarly treating appropriate standard solutions of acetone. Complete analysis of solutions of pure β-hydroxybutyric acid shows that only 86% of the theoretical amount of acetone is obtained. Allowance must be made for this in calculating the results.

Procedure for the estimation of total ketone bodies

When the possibility of an error of up to 5% can be tolerated a determination for total ketone bodies may be carried out (see Results). Sulphuric acid (8 ml.), water (40 ml.) and blood or urine filtrate (5 ml.) are boiled under reflux for 5 min. Potassium dichromate solution (2 ml.) and then water (10 ml.) are added to the boiling mixture and refluxed for a further 40 min. A distillate is collected which contains acetone derived from all ketone bodies. In calculating the results, the conversion of total ketone bodies into acetone is assumed to give a recovery of 91% based on the assumption that β-hydroxybutyric acid and acetone plus acetoacetic acid are present in the molar proportion of 2:1 (Weichselbaum & Somogyi, 1941).

RESULTS

All results were obtained with the Spekker absorptiometer (Ilford 601 filter) unless stated otherwise.

Colorimetric estimation of acetone

In a preliminary experiment it was found that on distillation 99% of acetone present could be collected in the first 4 ml. of distillate and the remainder in the next 7 ml. The procedure of Greenberg & Lester (1944) was modified so that 15 ml. of reagent, acetone distillate and washings was shaken with 6 ml. of carbon tetrachloride for extraction. To obtain reproducible readings it was necessary that the distribution of 2:4-dinitrophenylhydrazone and -hydrazine reached equilibrium between the aqueous–acid solution and the carbon tetrachloride. This condition was arrived at after shaking for 150 min. at 180 vibrations/min.

Greenberg & Lester (1944) showed that the amount of acetone-2:4-dinitrophenylhydrazone extracted by the carbon tetrachloride depended on the volumes and strengths of the fluids involved. As the acidity of the phenylhydrazine solution increased so the extraction of the hydrazone decreased. In the present work it was found that increased acidity shortened the shaking time required to reach equilibrium but increased the optical density of the blanks by causing more hydrazine to be extracted. The concentrations used

in the method represent a balance between rapid extraction on the one hand and high blanks on the other.

Maximum absorption of acetone-2:4-dinitrophenylhydrazone occurred at 350 mμ, and a second absorption peak was found at 410 mμ. Present results agree with those of Lester & Greenberg (1948), who found the absorption at 350 mμ approximately four times that at 420 mμ. Readings obtained with standard solutions of acetone equivalent to 0–80 mg./100 ml. of blood showed that Beer's Law was strictly followed over this range. Reproducibility of readings was within ± 1 %. With 0·1 ml. samples the method is sensitive to 0·03 mg./100 ml. of blood, the Unicam SP. 500 spectrophotometer being used at 350 mμ.

Distillation of acetone

Shipley & Long (1938) have reported a loss of 15 % when acetone is distilled. Greenberg & Lester (1944) and Michaels *et al.* (1951) criticized the use of distillation because of this risk. With the present apparatus and procedure it is possible to reflux and distil acetone without loss. A stemless dropping funnel was used so that no acetone was trapped on distillation. It was found that the capillary delivery tube (Fig. 1) forced the distillate to occupy the whole of the bore, ensuring complete washing out of acetone. It was essential for the condenser tube to be kept grease-free in order to obtain a regular flow of distillate.

Known quantities of acetone were refluxed for 10 min. under the conditions previously described. Acetone in the distillates was estimated colorimetrically; the results are summarized in Table 1.

Oxidation of β-hydroxybutyric acid

Shaffer & Marriott (1913–14), Van Slyke (1917) and Weichselbaum & Somogyi (1941) showed that variations in concentration of sulphuric acid and potassium dichromate affected the final yield of acetone. High concentrations of either of these reagents diminished the yield of acetone, but, on the other hand, the reaction was prolonged if the concentrations were very low. In recent methods there is a marked lack of correlation between the conditions employed and the results obtained for the oxidation of β-hydroxybutyric acid. Weichselbaum & Somogyi (1941), using a reaction mixture of 60 ml. volume, obtained 85 % conversion of β-hydroxybutyric acid into acetone in 40 min. Greenberg & Lester (1944), using the same concentrations of reagents, but in a much smaller volume of 3·6 ml. of solution, obtained 86 % conversion and reported that the reaction was complete in 10 min. Using approximately the same concentrations, Michaels *et al.* (1951) claimed 100 % conversion when the reaction mixture was autoclaved in screw-capped bottles at 15–20 lb./in.^{2} pressure. On the other hand, Thin & Robertson (1952), using the microrefluxing apparatus of Greenberg & Lester could obtain only a 60 % yield of acetone; alteration of the concentration of chromic acid, the time of oxidation or the concentration of β-hydroxybutyric acid failed to increase the percentage recovery.

To ensure reproducible results in the estimation of β-hydroxybutyric acid, the reagents must remain in constant concentration throughout the period of oxidation. In the present method, this has been achieved by: (i) refluxing the solutions; (ii) ensuring

Table 1. *Recovery of ketone bodies (expressed as acetone equivalents)*

Material added	Medium	Quantity of material added (range) (mg./100 ml.)	Quantity of material recovered (%)			No. of tests
			Mean	Range	S.E.	
		(*a*) Pure solutions				
Acetone	Water	11·3–30·8	99·8	98·5–101·6	0·17	14
β-Hydroxybutyric acid (as Ca–Zn salt)	Water	16·0–31·5	85·9	85·2–86·8	0·16	11
β-Hydroxybutyric acid (as sodium salt)	Water	31·6	85·75	85·5–86·0	—	2
		(*b*) Blood and urine				
Acetone	Blood	14·0–42·5	99·6	98·7–100·0	0·20	9
Acetone	Urine	24·3–44·9	99·6	98·2–100·8	0·21	9
Acetoacetic acid (as sodium salt)	Blood	16·5–50·9	98·3	97·5–99·3	0·22	9
Acetoacetic acid (as sodium salt)	Urine	24·5–51·8	98·1	97·2–98·7	0·19	9
β-Hydroxybutyric acid (as Ca–Zn salt)	Blood	16·1–42·7	99·7*	98·0–101·3	0·27	9
β-Hydroxybutyric acid (as Ca–Zn salt)	Urine	14·8–47·3	99·7*	98·6–100·9	0·21	9

* The conversion factor 85·9 % has been used in calculating this acetone equivalent (see text).

an excess of reagents, so that the oxidation of β-hydroxybutyric acid and other reducing substances present in biological fluids does not influence their effective concentrations; (iii) adding the reagents to the reaction mixture as quickly as possible.

Because the conversion of β-hydroxybutyric acid into acetone is incomplete, the common practice of estimating 'total ketone bodies' as one entity leads to inaccuracy, since the assumption is made that the ratio of the fraction of β-hydroxybutyric acid to that of acetone plus acetoacetic acid is 2:1 (Weichselbaum & Somogyi, 1941). Crandall (1940) has reported ratios as high as 15:1, and during the present work ratios have been found varying from 1:1 to 20:1. Accuracy can be attained only by estimating β-hydroxybutyric acid separately.

Time required for complete oxidation of β-hydroxybutyric acid. A series of replicate experiments was performed, with calcium zinc DL-β-hydroxybutyrate, in which the time of refluxing with potassium dichromate and sulphuric acid was varied from 10 to 75 min. before collection and determination of acetone in the distillates. The results showed that 30–40 min. was required for completing the reaction under present conditions and they agree with those of Weichselbaum & Somogyi (1941).

Percentage yield of acetone from β-hydroxybutyric acid. Aqueous solutions of different concentrations of calcium zinc DL-β-hydroxybutyrate were refluxed with acid dichromate for 40 min. The acetone formed was distilled and determined. A mean recovery of 85·9% of the theoretical quantity of acetone obtainable from β-hydroxybutyric acid was found (Table 1). When the sodium salt of DL-β-hydroxybutyric acid (British Drug Houses Ltd.) was used as a standard, similar results were obtained, demonstrating that the low results of Thin & Robertson (1952), who used a preparation from the same source, are unlikely to be due to its impurity.

Application of the method to blood and urine

Recovery experiments were carried out with standard solutions of ketone bodies added to blood and urine (Table 1). The mean recovery of acetone from blood and from urine was 99·6%.

The results with sodium acetoacetate (Table 1) show an invariable loss of nearly 2% of the acetoacetate added. The mean recoveries of 98·3% (S.E. ±0·22) and 98·1% (S.E. ±0·19) for blood and urine respectively were significantly different from 100% ($P < 0·001$). Subsequent work showed that the deproteinization technique was not at fault and that the presence of either blood or urine was necessary to cause the loss. In addition, the loss occurred only when the added acetoacetate was present as the sodium salt and did not occur with the ethyl ester.

When known quantities of calcium zinc DL-β-hydroxybutyrate were added to blood and urine a mean recovery of 99·7% (the conversion factor 85·9% being used) was obtained, which was not significantly different from 100% (P 0·4–0·2) (Table 1).

A series of estimations on samples of normal human blood was undertaken to show that the method did not give elevated values for the normal ketone-body level. The results, 0·3–0·6 mg. of total ketone bodies (expressed as acetone)/100 ml., were of the same order of magnitude as the results of Weichselbaum & Somogyi (1941) and Greenberg & Lester (1944). The latter authors found it necessary to introduce an extra period of refluxing with stronger dichromate in order to oxidize interfering substances more completely; in the present method this was found to be unnecessary.

Replicate determinations for ketone bodies were carried out on 0·1 ml. portions of a sample of blood to test the reproducibility of the method. At a ketonaemic level of 40 mg. (expressed as acetone/100 ml.) the standard deviation for the estimation of acetone plus acetoacetic acid was ±0·18 mg./100 ml. (12 determinations) and for the estimation of β-hydroxybutyric acid ±0·16 mg./100 ml. (9 determinations). These results were obtained with the Unicam SP. 500 spectrophotometer at 410 mμ.

Interference

Greenberg & Lester (1944) concluded that sugars and lactic acid, on treatment with sulphuric acid and dichromate, did not yield substances reacting with 2:4-dinitrophenylhydrazine. In the present method, only volatile substances such as aldehydes and ketones are able to interfere. Experiment showed that acetaldehyde caused interference which could not be eliminated completely. Prolonged shaking with the standard quantity of sodium hydroxide for 40 min. reduced the interference by half. When formaldehyde was treated similarly its interference was eliminated. Ethanol, which, if present, is distilled unchanged from acid solution in the acetone plus acetoacetic acid fraction, interferes only in the estimation of total ketone bodies by its reaction with the dichromate to form acetaldehyde.

When solutions of acetone-2:4-dinitrophenylhydrazone in carbon tetrachloride are shaken with sodium hydroxide solution, as described in the method, the fraction extracted by the alkali imparts a purple–red colour to this solution. On the other hand, the 2:4-dinitrophenylhydrazones of acetaldehyde and formaldehyde impart an orange–red colour to the alkaline layer. The occurrence of this colour is an indication of the presence of these aldehydes.

SUMMARY

1. A method is described for the accurate quantitative analysis of ketone bodies from small samples (0·1 ml.) of blood and urine.

2. The method is based on the conversion of all ketone bodies into acetone, on the reaction of acetone with 2:4-dinitrophenylhydrazine and on subsequent colorimetric determination of acetone-2:4-dinitrophenylhydrazone in carbon tetrachloride.

3. A combined reflux and distillation apparatus has been designed which ensures reproducibility in the estimation of β-hydroxybutyric acid and allows the separate and direct estimation of acetone plus acetoacetic acid and of β-hydroxybutyric acid from one sample.

4. At a ketonaemic level of 40 mg./100 ml., the standard deviation of an individual reading is less than $\pm 0{\cdot}2$ mg./100 ml.

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The Mechanism of Action of Mercurial Preparations on Transport Processes and the Role of Thiol Groups in the Cell Membrane of Renal Tubular Cells*

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The powerful diuretic action of mercurial preparations and investigation of the mechanism of their action *in vivo* have led to the generally accepted view that these substances directly or indirectly interfere with the reabsorption of sodium chloride and water by the tubular cells of the kidney (Schwartz & Wallace, 1951). It is also agreed that mercurial preparations exert their effect by inhibiting SH groups of some essential cellular enzymes (see, for example, Barron & Singer, 1945; Barron, 1951), but no clear-cut evidence is so far available concerning the localization of their action and detailed biochemical mechanism. Mudge (1951) observed that mercuric chloride (and also meralluride, an organic mercurial) at appropriate concentrations *in vitro*, inhibits the uptake of K^+ ion and loss of water by slices of kidney cortex which have previously been leached in isotonic sodium chloride at 0°, without, however, affecting the oxygen uptake of the tissue. A statement to the same effect has been made by Robinson (1954).

* A preliminary account was presented at the International Conference on Nervous trophic and active transport, Prague, August, 1956.

These results appeared to suggest therefore that mercurials interfere with the active transport of electrolytes in kidney-cortex slices, some authors maintaining that the primary effect is on Cl^- ion transport (Schwartz & Wallace, 1951; Axelrod & Pitts, 1952).

In this paper evidence is given for the view that mercurials interfere with the permeability of the membrane of tubular cells by increasing passive influx of Na^+ ion, Cl^- ion and water into the cells, without interfering with the active extrusion of Na^+ ion. Furthermore, evidence will be presented suggesting that the state of SH groups of proteins of the cellular membrane is a controlling factor in permeability. No evidence was found suggesting a direct effect of mercurials on Cl^- ion transport *per se*.

METHODS

The experimental procedure used has already been described in some detail (Cort & Kleinzeller, 1956) and will only be summarized here. Unless otherwise stated, slices of kidney cortex of normal rabbits (the uppermost slice being rejected) were leached in 0·154 M-NaCl at 0° for 2·5 hr., and samples of slices (usually 100–200 mg. wet wt.) were then

incubated at 25° in 3 ml. of phosphate–saline containing a mercurial preparation or other additions, and in most experiments also substrate (0·005 M-α-oxoglutarate). Slices incubated in the same medium without mercurials (or other additions) served as controls.

The contents of water, Na^+ and K^+ ions were estimated in the slices previously described (Cort & Kleinzeller, 1956). Where the content of Cl^- ion was also estimated, the dry tissue was crushed, extracted with 0·2 N-H_2SO_4 for about 5 days, and Na^+, K^+ and Cl^- ions were estimated in the extract, the last by amperometric titration, a calomel-dropping–Hg electrode system, a 5% KNO_3 bridge and 0·02 N-$HgNO_3$ being used for microtitration (Kolthoff & Miller, 1941; Kubie, in preparation). This method is accurate to a total Cl^- ion sample of about 2 μ-equiv. in 1 ml. of solution. Wherever a significantly lower amount was found, it has been designated as 0.

The results are expressed in m-equiv. of the appropriate ion/100 g. of dry solids. Samples of tissue were analysed at the following intervals: initial value (I), after leaching (T_0), and at various intervals after incubation, the subscript of T indicating minutes of incubation. The same procedure was adopted where other tissues were used.

In experiments with ^{24}Na, the procedure was similar to that described earlier (Cort & Kleinzeller, 1956). Tissue slices were leached in isotonic NaCl with or without ^{24}Na present. The difference between the values of Na^+ ion at I and T_0 was used to estimate the relative passive penetration of Na^+ ion into the cells. Samples of slices, leached in labelled NaCl, were then quickly washed in non-isotopic NaCl, and transferred to vessels containing 2 ml. of phosphate–saline with or without mercurial, containing 0·005 M-α-oxoglutarate as substrate. After incubation for 10 min. at 25° the slices were removed, blotted and weighed, and dried at 110° over P_2O_5 for 5 hr.; the dry solids were weighed and digested for 15 min. at 100° with 1 ml. of conc. HNO_3. The Na^+ and K^+ ion content in these digests was then estimated. Portions of the filtered medium were used for measuring radioactivity. Measurements were made with a low-voltage liquid counter of approximately 4π geometry. From these measurements the relative inward and outward fluxes of ^{24}Na (M_i and M_o) were estimated. At the same time, relative $M_{i,\,Na}$ was estimated directly by leaching the slices in non-labelled NaCl, incubating them in phosphate–saline labelled with ^{24}Na, and measuring the specific activity of ^{24}Na in the tissue. The results are expressed as relative fluxes (M_i and M_o) in 10^7 counts/sec./100 g. of tissue solids.

Oxygen uptake of tissue slices was measured at 37° by the Warburg technique, 3 ml. of phosphate–saline, or a specific medium as described in the text, being used. The results are expressed as Q_{O_2}.

In some experiments the non-protein and protein N in the medium after incubation of the slices were estimated as follows: the medium was centrifuged, and 0·5 ml. of 10% trichloroacetic acid was added to a 2 ml. portion. After standing overnight, the samples were centrifuged, and 2 ml. of the supernatant was digested with the Cu–Se catalyst described by Sober, Mayer & Gottfried (1944). The sediment was washed once with 2 ml. of 5% trichloroacetic acid, centrifuged, then dissolved in 0·5 ml. of N-KOH and digested. Ammonia was then estimated by the micro-diffusion technique (Conway, 1947). The results are expressed in μg. of N/10 mg. of dry solids.

All values are the mean of two individual analyses, which generally did not differ by more than 3–5%. All experiments were repeatedly confirmed; representative results only are presented.

Most of the experiments described were carried out with either $HgCl_2$ ($4–5 \times 10^{-4}$ M, final concn.) or $1·45 \times 10^{-3}$ M (final concn.) Esidrone (i.e. theophyllino-mercuri-monooxypropylamido sodiumquinolinate, Ciba, Basel, Switzerland); in some of the experiments $2·8 \times 10^{-3}$ M (final concn.) Novurit (i.e. sodium N-(γ-hydroxymercuri-β-methoxy)-propylcamphoramate, SPOFA, Prague, Czechoslovakia), or p-chloromercuribenzoate (10^{-4} M) was used with essentially the same results.

Reduced glutathione and p-quinone were commercial preparations; α-oxoglutaric acid was obtained from Dr J. Kocourek, dehydroascorbic acid was prepared by Dr J. Liebster.

RESULTS

Effect of mercurials added during incubation on the transport of electrolytes and water. Mudge (1951) observed in experiments of 30 min. duration that mercuric chloride at a concentration of 5×10^{-4} M (and also at higher concentrations), and also meralluride 10^{-3} M, inhibited the transport of water and K^+ ion, without commensurably affecting the O_2 uptake. Similar results concerning water were obtained by Robinson (1954) with another mercurial. If, however, the effect of Esidrone (or Novurit) is examined at shorter time intervals, it is found that during the first 10 min. of incubation the slices lose water and Na^+ ion at a rate only slightly lower than the controls without mercurial added,

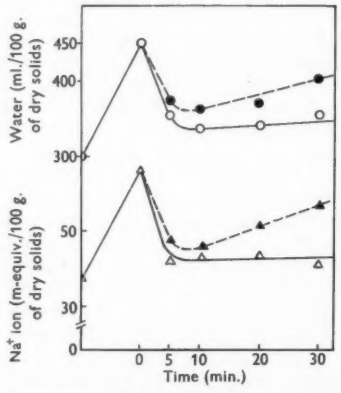

Fig. 1. Time curve of effect of Esidrone added during incubation on transport of Na^+ ion and water. Slices were leached for 2·5 hr. at 0° in 0·154 M-NaCl and then incubated at 25° in 3 ml. of phosphate–saline containing 0·005 M-α-oxoglutarate, without or with $1·45 \times 10^{-3}$ M-Esidrone. —, Controls; – – –, Esidrone. The top set of curves represent water (ml./100 g. of dry solids), the lower Na^+ (m-equiv./100 g. of dry solids).

Table 1. *Effect of incubation with Esidrone on the transport of water, Na^+, K^+ and Cl^- ions in kidney-cortex slices*

Slices were leached for 2·5 hr. at 0° in 0·154 M-NaCl and then incubated at 25° in 3 ml. of phosphate–saline containing 0·005 M-α-oxoglutarate and Esidrone. All values are in ml. or m-equiv./100 g. of dry solids.

Mercurial	Time interval (min.)	Water (ml.)	Na^+	K^+ (m-equiv.)	Cl^-
Nil	Initial	287	25·7	30·7	17·9
Nil	0	377	53·6	14·7	54·4
Nil	10	317	34·5	18·2	32·8
Esidrone ($1·45 \times 10^{-3}$ M)	10	319	37·4	17·6	35·3
Nil	30	302	33·2	25·4	30·4
Esidrone ($1·45 \times 10^{-3}$ M)	30	330	43·7	14·7	39·9

Table 2. *Effect of incubation with mercurials on the loss of nitrogenous substances by kidney-cortex slices*

Slices were leached for 2·4 hr. at 0° in 0·154 M-NaCl and then incubated in 3 ml. of medium A_2 (Robinson, 1949) containing 0·005 M-α-oxoglutarate. NPN, Non-protein N; PN, protein N. All values in μg./10 mg. of dry solids in the medium after incubation.

Mercurial	Time interval (min.)	Expt. 1 NPN (μg.)	Expt. 1 PN (μg.)	Expt. 2 NPN (μg.)	Expt. 2 PN (μg.)	Expt. 3 NPN (μg.)	Expt. 3 PN (μg.)
Nil	10	33·8	21·3	11·6	18·5	7·7	13·7
$HgCl_2$ (5×10^{-4} M)	10	25·6	25·4	10·2	19·2	8·6	14·5
Esidrone ($1·45 \times 10^{-3}$ M)	10	—	—	—	20·4	7·0	14·9
Nil	30	29·5	20·2	12·0	22·3	16·0	23·2
$HgCl_2$ (5×10^{-4} M)	30	36·6	64·5	19·7	44·2	18·8	39·6
Esidrone ($1·45 \times 10^{-3}$ M)	30	—	—	—	28·6	36·6	26·5

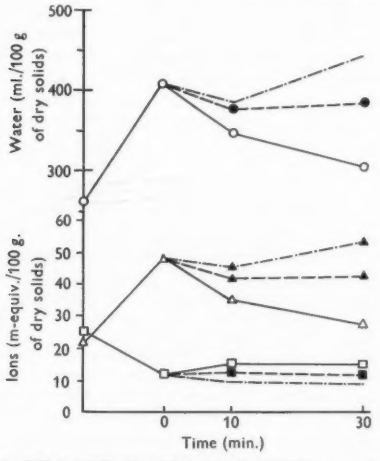

Fig. 2. Effect of $HgCl_2$ added during incubation on transport of cations and water. Conditions were as described for Fig. 1. —, Without $HgCl_2$; – – –, $3·7 \times 10^{-4}$ M-$HgCl_2$; –·–·–, 5×10^{-4} M-$HgCl_2$. Top set of curves: water; middle set: Na^+ ion; bottom set: K^+ ion.

but take up water and Na^+ ion again during further incubation (Fig. 1); the movement of K^+ ion is reciprocal to that of Na^+ ion. The water, Na^+ and K^+ ion contents of the slices after 30 min. incubation differ little from, or may even be higher than, the T_0 values.

No appreciable inhibitory effect of Esidrone (at the concentration used) on the O_2 uptake of the tissue was found (Q_{O_2} values: controls, −15·2 without substrate, −21·4 with substrate; with added mercurial, −12·1 without substrate, −22·0 with substrate).

Although the time curve for the effect of mercuric chloride followed the same trend, the effect was less clear. Fig. 2 shows the effect of two concentrations of mercuric chloride (3·7 and 5×10^{-4} M) on the transport of water, Na^+ and K^+ ions.

It has been suggested (Schwartz & Wallace, 1951; Axelrod & Pitts, 1952) that mercurials may specifically affect the transport of Cl^- ion. As shown in Table 1, the values for Cl^- ion appear to follow Na^+ ion, and the changes of Cl^- ion are of the same order as Na^+ ion changes (see also Leaf, 1956). This result does not support a specific effect of mercurial on Cl^- ion transport, but does not exclude it. A stimultaneous movement of α-oxoglutarate anion may also have occurred under these conditions (see Whittam & Davies, 1953).

Effect of mercurials added during incubation on the loss of nitrogenous substances by slices of kidney cortex. It is known that kidney-cortex slices, when incubated in phosphate–saline, lose some protein nitrogen into the medium; such loss can be reduced in a slightly modified medium (see Robinson, 1949; Aebi, 1952). It will be seen from Table 2 that incubation of the slices in medium A_2 (Robinson, 1949), containing 0·005 M-α-oxoglutarate as substrate, and mercurial brings about a marked loss of protein

nitrogen between 10 and 30 min. incubation, as compared with controls without added mercurials. The increased breakdown of tissue protein in the presence of mercurials may contribute to the renewed uptake of electrolyte and water by the tissue after 10 min. incubation, as shown above.

Effect of mercurials added during leaching on the transport of cations and water by slices of kidney cortex. The results reported above suggested that mercurials affected a process other than active transport of cations (and water?) by tubular cells, since no effect was observed in the first 10 min. of incubation. Further evidence was provided by the following experiment: if slices of kidney cortex are leached in isotonic NaCl containing $1{\cdot}45 \times 10^{-3}$ M-Esidrone (the leaching temperature of 0° inhibiting active transport), the tissue takes up considerably more Na^+ ion and water, and loses more K^+ ion, than controls leached in NaCl alone. If the slices leached in NaCl and Esidrone are then incubated in phosphate–saline with or without substrate (the slices being quickly washed in NaCl before transferring them into vessels for incubation in order to remove adhering fluid containing mercurial, there being a residual component bound to tissue proteins), the rate of Na^+ ion and water loss, and the rate of K^+ ion uptake, do not differ from that of controls leached in NaCl, or may even be higher; however, a new steady state of the levels of Na^+ ion, water and K^+ ion in the tissue is established, the Na^+ ion and water content being higher, the K^+ ion content lower, than in the controls (Fig. 3). The same result was obtained with leaching in NaCl containing, for example, 4×10^{-4} M-mercuric chloride.

Since mercurials are firmly bound by the proteins of kidney cortex (Borghgraef & Pitts, 1956; Greif, Sullivan, Jacobs & Pitts, 1956; Leuschner, 1956),

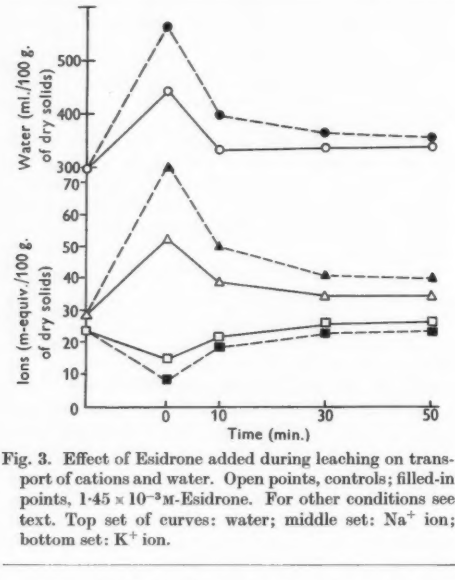

Fig. 3. Effect of Esidrone added during leaching on transport of cations and water. Open points, controls; filled-in points, $1{\cdot}45 \times 10^{-3}$ M-Esidrone. For other conditions see text. Top set of curves: water; middle set: Na^+ ion; bottom set: K^+ ion.

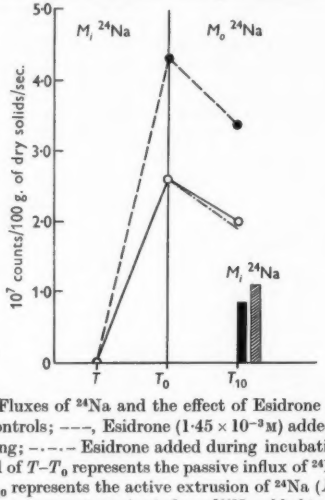

Fig. 4. Fluxes of ^{24}Na and the effect of Esidrone thereon. —, Controls; – – –, Esidrone ($1{\cdot}45 \times 10^{-3}$ M) added during leaching; –·–·– Esidrone added during incubation. The period of T–T_0 represents the passive influx of ^{24}Na (M_i); T_0–T_{10} represents the active extrusion of ^{24}Na (M_o). The columns represent passive influx of ^{24}Na added to separate groups of slices during incubation. Filled-in column, control; cross-hatched column, with Esidrone added during incubation. For details of conditions see text.

Table 3. *Effect of various mercurial preparations on the transport of cations and water in kidney-cortex slices during leaching*

Slices were leached at 0° for 2·5 hr. in 0·154 M-NaCl with or without a mercurial present. All values are in ml. or m-equiv./100 g. of dry solids.

Mercurial	Initial values			After leaching in NaCl			After leaching in NaCl with mercurial		
	Water (ml.)	Na^+ (m-equiv.)	K^+ (m-equiv.)	Water (ml.)	Na^+ (m-equiv.)	K^+ (m-equiv.)	Water (ml.)	Na^+ (m-equiv.)	K^+ (m-equiv.)
$HgCl_2$ (4×10^{-4} M)	276	19·4	23·7	400	47·0	13·1	478	53·0	12·0
Esidrone ($1{\cdot}45 \times 10^{-3}$ M)	317	24·5	30·4	438	52·2	11·3	512	66·0	8·3
p-Chloromercuribenzoate (10^{-4} M)	311	16·9	24·6	423	48·9	7·9	515	63·9	7·1

Table 4. *Effect of leaching in isosmolar sodium sulphate and sodium chloride on the transport of cations and water in kidney-cortex slices*

Slices of kidney cortex were leached at 0° for 2·5 hr. in 0·154 M-NaCl (0·308 osmolar), 0·1145 M-NaCl (0·229 osmolar) and 0·0764 M-Na_2SO_4 (0·229 osmolar). All values are in ml. or m-equiv./100 g. of dry solids.

Time	Leaching medium	Water (ml.)	Na^+ (m-equiv.)	K^+ (m-equiv.)
Initial	—	298	20·7	22·6
T_0	0·154 M-NaCl	390	51·7	9·7
T_0	0·1145 M-NaCl	432	51·6	12·1
T_0	0·0764 M-Na_2SO_4	368	48·8	8·2

this result is regarded as evidence that mercurials affect primarily the permeability of the tubular cell membrane for Na^+ ion and water.

The increased uptake of Na^+ ion and water by slices of kidney cortex was also observed with another mercurial preparation, *p*-chloromercuribenzoate, as shown in Table 3. The effect described appears therefore to be characteristic of mercuric compounds, inorganic and organic.

Effect of Esidrone on the fluxes of ^{24}Na. The experimental results reported above were confirmed in four experiments with ^{24}Na. The results of a representative experiment (Fig. 4) show that in the course of the experiment (lasting 10 min.) the addition of $1{\cdot}45 \times 10^{-3}$ M-Esidrone to the medium did not affect the active transport of Na^+ ion out of cells (M_o), while considerably increasing the passive inflow (M_i). Addition of Esidrone during leaching in NaCl at 0° greatly increased M_i, while the rate of extrusion of ^{24}Na from cells during 10 min. incubation at 25° was even higher than that of controls. This last observation suggests that the active transport of Na^+ ion out of cells is also a function of the concentration gradient across the cell membrane. Such a conclusion has also been reached in experiments in which the concentration gradient of Na^+ ion has been changed (Cort & Kleinzeller, 1957).

Effect of mercurials added during leaching on the transport of Cl^- *ion*. Since Schwartz & Wallace (1951) and Axelrod & Pitts (1952) have expressed the view, on the strength of experiments *in vivo*, that mercurials primarily affect the transport of Cl^- ion in the kidney, and since Koefoed-Johnsen & Ussing (1956) have reported experiments suggesting that another heavy metal (copper) decreases the permeability for Cl^- ion of the frog skin, further experiments were directed to elucidate the primary biochemical action of mercurials. The effect of mercurials on the transport of Cl^- ion was studied under conditions where the concentration gradient of Cl^- ion between tissue and medium was changed, without varying the concentration gradient of sodium. In these experiments SO_4^{2-} ion replaced Cl^- ion as the anion. The Na_2SO_4 solution used (0·0764 M) is hypotonic as compared with 0·154 M-NaCl solution, the Na^+ ion concentration being the same in both. Since the osmolarity of the medium is a determinant of water and cation transport during leaching (Robinson, 1950), as a preliminary to the study of the effect of mercurials the effect of the hypotonic Na_2SO_4 solution on these transport processes was examined. The results are presented in Table 4, in which changes of water and cation contents during leaching in 0·154 M-NaCl are compared with changes on leaching in 0·0764 M-Na_2SO_4 and 0·1145 M-NaCl, the latter solution being isosmolar with the Na_2SO_4 solution.

It will be seen that the uptake of water and Na^+ ion during leaching is less in the Na_2SO_4 solution than in the 0·154 M-NaCl solution. Such a result would be expected in a hypotonic solution containing an anion to which the cell membrane is far less permeable than Cl^- ion. The uptake of water due to reduced osmolarity may be assessed as follows: slices leached in 0·1145 M-NaCl took up an additional 42 ml. of water/100 g. of dry solids, as compared with slices leached in 0·154 M-NaCl. This value has to be compared with the uptake of 70 ml. of water in slices leached in Na_2SO_4.

The following procedure was used to examine the effect of mercurial on the transport of Cl^- ion: kidney-cortex slices were leached at 0° in either 0·154 M-NaCl or 0·0764 M-Na_2SO_4, with or without $1{\cdot}45 \times 10^{-3}$ M-Esidrone. After leaching the slices were incubated for 10 min. at 25° either in the usual phosphate–saline or in a modified medium in which all Cl^- ion was replaced by SO_4^{2-} ion; this medium is also somewhat hypotonic (245 m-osmolar as compared with 305 m-osmolar of the standard medium). The results of one such experiment are shown in Table 5.

It will be seen that Cl^- ion leaves cells practically completely on leaching in Na_2SO_4 solution, while Na^+ ion increases. The continued increase in cell Na^+ ion must be balanced by another anion. Our own results do not permit an answer to this question, but it may be suggested that protein degradation in the cold (Conway, Geoghegan & McCormack, 1955) might provide the additional anion equivalence.

In accordance with the low permeability of the cell membrane for SO_4^{2-} ion no appreciable effect of Esidrone on the uptake of water and Na^+ ion was

Table 5. *Effect of Esidrone, added during leaching, on transport of* Cl^- *ion in kidney-cortex slices*

Slices were leached for 2·5 hr. at 0° in either 0·154 M-NaCl or 0·077 M-Na_2SO_4, with or without $1·45 \times 10^{-3}$ M-Esidrone. Slices were then incubated for 10 min. at 25° either in 3 ml. of phosphate–saline of usual composition (Cl^- ion medium), or in 3 ml. of a phosphate–saline in which Cl^- ion was replaced by SO_4^{2-} ion (SO_4^{2-} ion medium); 0·005 M-α-oxoglutarate was present as substrate. All values are in ml. or m-equiv./100 g. of dry solids.

	Initial				T_0				Cl^- ion medium T_{10}				SO_4^{2-} ion medium T_{10}			
Leaching medium	Water (ml.)	Na^+	K^+ (m-equiv.)	Cl^-	Water (ml.)	Na^+	K^+ (m-equiv.)	Cl^-	Water (ml.)	Na^+	K^+ (m-equiv.)	Cl^-	Water (ml.)	Na^+	K^+ (m-equiv.)	Cl^-
—	302	32·6	30·1	19·8	—	—	—	—	—	—	—	—	—	—	—	—
NaCl	—	—	—	—	412	58·8	13·6	46·3	325	37·1	22·2	29·9	346	34·5	21·6	4·6
NaCl + Esidrone	—	—	—	—	475	78·4	10·3	59·6	393	48·4	17·8	42·9	391	51·8	14·0	4·0
Na_2SO_4	—	—	—	—	376	54·3	9·5	0	332	48·8	21·2	23·9	364	41·8	19·7	0
Na_2SO_4 + Esidrone	—	—	—	—	365	52·3	8·4	0	357	41·8	23·6	21·8	359	38·0	22·6	0

Table 6. *Transport of cations and water in kidney slices leached in the presence of Esidrone and incubated in phosphate–saline with and without* K^+ *ion*

Slices were leached at 0° for 2·5 hr. in 0·154 M-NaCl without and with $1·45 \times 10^{-3}$ M-Esidrone. Slices were then incubated at 25° in 3 ml. of phosphate–saline (6·25 m-equiv. of K^+ ion/l.) or phosphate–saline in which K^+ ion was replaced by an equivalent amount of Na^+ ion; 0·005 M-α-oxoglutarate was present as substrate. All values are in ml. or m-equiv./100 g. of dry solids.

		Time										
		Initial			T_0			T_{10}			T_{30}		
Leaching medium	K^+ ion concn. in incubation medium (m-equiv./l.)	Water (ml.)	Na^+ (m-equiv.)	K^+	Water (ml.)	Na^+ (m-equiv.)	K^+	Water (ml.)	Na^+ (m-equiv.)	K^+	Water (ml.)	Na^+ (m-equiv.)	K^+
NaCl	6·25	313	23·3	21·9	391	45·0	11·1	309	31·9	19·0	294	29·8	23·4
NaCl + Esidrone	6·25	—	—	—	590	70·6	10·7	417	56·3	16·1	369	47·3	16·2
NaCl	0	—	—	—	—	—	—	323	41·7	10·6	285	42·1	10·5
NaCl + Esidrone	0	—	—	—	—	—	—	442	65·2	8·4	391	59·2	8·3

observed on leaching in 0·0764 M-Na_2SO_4. This result also suggests that mercurials do not affect the extracellular space of the slices and that mercurials during leaching act directly on tubular cells.

Slices leaching in Na_2SO_4 and then incubated in the standard phosphate–saline lose water and re-accumulate K^+ ion at approximately the same rate as those leached in NaCl, the extrusion of Na^+ ion being considerably slower. However, slices leached in Na_2SO_4 containing Esidrone extrude Na^+ ion and re-accumulate K^+ ion, while their content of water does not change. This is the third instance where a dissociation between the transport of water and Na^+ or K^+ ion has been observed (see Cort & Kleinzeller, 1956). Concerning the movement of Cl^- ion, it must be pointed out that Cl^- ion is lost on leaching in Na_2SO_4 and re-accumulates at the same rate on incubation in standard phosphate–saline, whether or not the slices were leached in Esidrone.

Slices leaching in either NaCl or Na_2SO_4, with or without Esidrone, and incubated in the SO_4^{2-} ion medium, showed the following behaviour: slices leached in NaCl with or without Esidrone lost water and Na^+ ion, and re-accumulated K^+ ion in the same manner as in the standard phosphate–saline. At the same time a loss of Cl^- ion occurred in accordance with its concentration gradient. Slices leached in Na_2SO_4 with or without Esidrone extruded Na^+ ion and re-accumulated K^+ ion without appreciable change in water content. It should be mentioned that the rate of O_2 uptake is the same whether phosphate–saline or the SO_4^{2-} ion medium is used (Q_{O_2} in standard phosphate–saline: $-25{\cdot}2$; Q_{O_2} in the SO_4^{2-} ion medium: $-25{\cdot}3$). It will be noted that brain-cortex slices also do not show an impaired metabolic activity when incubated in a Cl^- ion-free medium (Thomas & McIlwain, 1956). It thus appears that in kidney or brain cortex such metabolic activities as oxidation of substrates do not require Cl^- ion.

The results reported above are difficult to reconcile with the suggestion that mercurials affect a specific transport process for Cl^- ion, especially that part of the experiment where slices were leached and incubated in the appropriate SO_4^{2-} ion media.

Effect of Esidrone added during leaching on the transport of K^+ *ion.* It might be concluded from the work of Mudge (1951, 1953) that mercurials acted primarily on the transport of the K^+ ion. If that were the case, slices leached in NaCl and mercurial might behave differently from controls when incubated in a medium not containing K^+ ion. It has been shown previously (Cort & Kleinzeller, 1956) that slices leached in NaCl and then incubated in a modified phosphate–saline without K^+ ion lose water, the cation content remaining constant. Slices leached in NaCl and Esidrone behave in the same way (Table 6). The loss of water during incubation does not appear to be dependent on the intracellular concentration of K^+ ion within the limits of about 5 m-equiv./100 g. of dry solids (lowest values for K^+ ion in slices leached in NaCl and Esidrone) and about 30 m-equiv./100 g. of dry solids (normal slices).

Effect of mercuric chloride added during leaching on the transport of cations and water in other tissues. In view of the drastic effect of mercurials on renal function it was considered of interest to examine the effect of mercuric chloride during leaching on the transport of cations and water in various tissues. The results are summarized in Table 7. It will be seen that leaching in NaCl containing 5×10^{-4} M-mercuric chloride increases the Na^+ ion and water content in brain and heart, as in kidney, but does

Table 7. *Effect of* 5×10^{-4} M-*mercuric chloride on the transport of cations and water during leaching of various tissues from the guinea pig*

Slices of tissue were leaching at 0° for 2·5 hr. in 0·154 M-NaCl with and without 5×10^{-4} M-$HgCl_2$. All values are in ml. or m-equiv./100 g. of dry solids. Standard error is listed for those values where five experiments were performed. The figures otherwise presented are averages. The number of experiments is given in parenthesis.

	Initial values			After leaching in NaCl			After leaching in NaCl + $HgCl_2$		
Tissue	Water (ml.)	Na^+ (m-equiv.)	K^+	Water (ml.)	Na^+ (m-equiv.)	K^+	Water (ml.)	Na^+ (m-equiv.)	K^+
Kidney (2)	354	21·3	29·6	431	42·4	13·1	544	63·5	9·2
Liver (2)	245	10·4	29·0	524	76·5	6·6	496	73·8	5·6
Brain cortex (5)	501 ±14	36·7 ±3·0	31·8 ±3·6	887 ±34	105·6 ±5·7	6·9 ±0·9	954 ±17	116·9 ±7·3	3·2 ±0·5
Diaphragm (2)	302	19·8	28·8	570	71·2	6·9	558	75·8	4·1
Somatic muscle (gracilis) (5)	368·5 ±15	16·6 ±1·3	38·2 ±1·7	577 ±11	79·8 ±3·9	8·3 ±0·8	588 ±27	74·8 ±4·3	4·9 ±0·9
Myocardium (5)	368 ±2	14·3 ±0·7	22·3 ±0·6	477 ±8	66·1 ±1·6	5·9 ±0·3	523·5 ±18	72·5 ±2·1	3·3 ±0·4

Table 8. *Effect of various concentrations of* p-*quinone during leaching on the transport of cations and water in kidney-cortex slices*

Slices were leached at 0° for 2·5 hr. in 0·154 M-NaCl with or without quinone. All values are in ml. or m-equiv./100 g. of dry solids.

p-Quinone concn. (M)	Time	Water (ml.)	Na^+ (m-equiv.)	K^+ (m-equiv.)
—	Initial	291	21·2	21·4
—	T_0	397	47·3	12·1
5×10^{-4}	T_0	442	50·2	11·4
10^{-3}	T_0	435	53·0	11·7
2×10^{-3}	T_0	477	57·4	6·5

not affect the Na^+ ion and water content in liver or striated muscle (gracilis and diaphragm). However, in all tissues examined mercuric chloride brought about a decrease of the K^+ ion content.

Effect of p-*quinone on the transport of cations and water during leaching.* Since *p*-quinone reacts with SH groups (see Barron, 1951), its effect on the transport of cations and water in kidney-cortex slices during leaching was examined. As shown in Table 8, *p*-quinone in concentrations 5×10^{-4}–2×10^{-3} M brings about a considerable increase of Na^+ ion and water content of the slices, as compared with slices leached in NaCl alone. Further experiments, not reported in detail here, showed that the effect of *p*-quinone during leaching is partly reversible on incubation in a similar manner to the effect of mercurials.

DISCUSSION

Effect of mercurials on electrolyte transport in kidney-cortex cells. The results reported above indicate that mercurials (organic and inorganic) increase the passive movement of Na^+ ion, Cl^- ion and water into renal tubular cells, leaving unaffected active transport of Na^+ ion out of the cells. It may also be argued that the changes depicted in Figs. 1 and 2 could be the result of a relatively slow penetration of mercurials into the cells. However, two facts militate against such an interpretation of the results. First, the results obtained on leaching slices in sodium chloride containing Esidrone (Fig. 3) or mercuric chloride: since it is known that mercurials are firmly bound by proteins of kidney tissue and cannot be removed, e.g. by dialysis (Greif *et al.* 1956), this experiment appears to exclude the possibility of mercurials affecting active-transport processes. Also our experiments with ^{24}Na in the medium did not show any change of sodium efflux as compared with controls. Secondly, it should be noted that a similar, if somewhat more rapid, effect on the movement of Na^+ ion and water during incubation was obtained with nearly equivalent amounts of either mercuric chloride or Esidrone, in spite of considerable differences of molecular weight (equivalence being calculated on the basis of mercury free to react with SH groups; such calculation appears to be justified in view of the fact that in experiments both *in vivo* and *in vitro* it has been reported that the C–Hg bond in organic mercurials is not broken by the metabolic activity of the whole organism (Weiner & Müller, 1955) or tissue homogenate (Leuschner, 1956). The uptake of Na^+ ion, Cl^- ion and water by the slices after the first 10 min. of incubation appears therefore to be the result of an irreversible damage to the cells by the high concentrations of mercurials present, accompanied by a release of protein nitrogen into the medium, rather than the result of inhibition of active transport. In all experiments it was found that mercurials added during leaching brought about a decrease in cell K^+ ion, whether or not Na^+ ion and water content was affected. This phenomenon was not studied further, but it would appear from the results of Mudge (1953) that mercurials may have affected the amount of non-exchangeable K^+ ion in the tissue.

The view that mercurials primarily affect the transport of Cl^- ion (Schwartz & Wallace, 1951; Axelrod & Pitts, 1952) is based essentially on observation of the effect of mercurials on excretion of Cl^- ion and chloride balance in whole animals and humans. This conclusion must be taken as indirect since the experimental approach precluded any information about cellular changes in the kidney. Not only have our own results failed to confirm this view, but it is clear from the experiments in which SO_4^{2-} ion replaced Cl^- ion in the medium, that in fact Cl^- ion and Na^+ ion can move in opposite directions across the cell membrane at a time when there is a clear effect of mercurials on Na^+ ion penetration. These results also provide further evidence for the view (Whittam, 1956) that in kidney-cortex cells Cl^- ion is passively transported. The only clear effect on electrolytes that we could demonstrate for mercurials was on the diffusion resistance of the tubular-cell membrane for Na^+ ion.

Considerable evidence has been accumulated showing that mercurials affect metabolic processes of the cells by combining with SH groups of cell enzymes. Since it has been here shown that mercurials primarily decrease the resistance of the

cell membrane towards passive movements of Na^+ ion and water, it may be concluded that such SH groups of the protein component of the cellular membrane are of importance in the regulation of cell permeability. In view of the fact that only kidney-cortex tissue and, to a lesser extent, myocardium and brain cortex appear to be affected by mercurials, it would follow that these tissues may contain in their cell membranes a protein component capable of combining specifically with mercurial preparations. It should be mentioned that in myocardium radioactive mercury is fixed on the cellular membrane, as shown by historadioautography (Koshtoyants, personal communication).

Mercurials are most often used clinically in congestive cardiac failure, but aside from kidney and brain, the myocardium is the only other significant tissue (including somatic muscle) whose transport activity is specifically depressed by mercurials.

Mechanism of diuretic action of mercurials. Recently, Borghgraef & Pitts (1956) provided evidence that the main site of action of mercurial diuretics is the cells of the renal cortex, since 80 % of the mercurial injected was localized in this tissue. How do the concentrations used in our experiments compare with those required for a physiological or clinical effect in the intact animal? Since diuresis is produced clinically by the intravenous administration of 2 ml. (0·216 m-mole) of Esidrone, and since both average human kidneys are 600–700 g., of which about two-thirds is cortex, one may roughly calculate that the concentration of mercurial/l. of cortical-tissue water was of the order of 5×10^{-4} M. This figure is about one-third of the concentration used by us, but it must be pointed out that the effect of mercurial on tissue slices can be observed with far lower concentrations, but that for purposes of greater constancy and statistical significance we wished to measure a maximal, but not too toxic, effect. Mudge (1951) found a clear effect of meralluride on kidney-cortex slices at a concentration as low as 3×10^{-4} M. The overwhelming amount of mercurial in kidney was found in the soluble fraction of the disintegrated tubular cells (Greif *et al.* 1956). In view of the very high activity of mitochondria as compared with whole cells in the active transport of cations (see Davies, 1954), and the relatively small proportion of mercury bound by the mitochondrial fraction (Greif *et al.* 1956), these results would also be consistent with the view that mercurials affected a process other than the active extrusion of Na^+ ion. By histochemical methods no changes of the morphology of tubular-cell mitochondria have been found after administration of mercurial (Lojda, personal communication). The main mechanism of action of mercurials *in vivo* appears therefore to be a change in the permeability of the cell membrane of tubular cells, thus increasing the passive movement of Na^+ ion, Cl^- ion and water into cells, and resulting in a reduction of total reabsorbed Na^+ ion, Cl^- ion and water.

It has been previously suggested (Cort & Kleinzeller, 1956) that the active extrusion of Na^+ ion from tubular cells is primarily localized at or near the basal membrane of the tubular cells. Since this membrane is in most direct contact with the blood vessels through which mercurials are transported in the kidney and into which the flow of Na^+ ion and water from tubular urine is directed, it would appear to be in accord with available evidence to suggest that this is also the place where mercurials exert their effect, by chemical reaction with SH groups of the membrane proteins. Since mercurials and mercury in blood are bound to albumin (Edsall, 1954), only an insignificant fraction of serum mercury would be filtered.

Role of SH *groups of membrane proteins in cellular permeability.* The results obtained with *p*-quinone provide further evidence, in addition to that obtained with mercurials, that SH groups of proteins of the cell membrane play a role in the permeability of tubular cells for cations and Cl^- ion. So far, no conclusive results have been obtained with dehydroascorbic acid as an acceptor of hydrogen from the SH groups, in conditions under which *p*-quinone was effective, although it is known to oxidize SH groups of, for example, glutathione, in a non-enzymic reaction (Szent-Györgyi, 1928). An explanation for this may be sought in the lower redox potential of the dehydroascorbic–ascorbic acid system as compared with the *p*-quinone–quinol system. No consistent results have been obtained with reduced glutathione (10^{-3} M) added during leaching, although in most of the experiments the Na^+ ion content in the slices was slightly lower, the K^+ ion content somewhat higher, than in the controls without glutathione.

Davson (1943) and LeFevre (1948) provided evidence suggesting that SH groups in the membrane of erythrocytes also played a role in the permeability of these cells. It would appear therefore that the state of SH groups in membrane proteins (which is a reflexion of the redox potential at the cell surface) influences cell permeability and is thus of more general importance for cell metabolism. Such conclusion is in full agreement with that of Barron (1951).

SUMMARY

1. The effect of mercuric chloride and organic mercurials [Esidrone (CIBA), Novurit (SPOFA), *p*-chloromercuribenzoate] and *p*-quinone on the transport of Na^+, K^+ and Cl^- ions and water by slices of rabbit kidney cortex was investigated.

2. Addition of $1{\cdot}45 \times 10^{-3}$M-Esidrone, or $2{\cdot}8 \times 10^{-3}$M-Novurit, affected only slightly the decrease of Na^+ ion and water, and increase of K^+ ion, during 10 min. incubation at 25°. Further incubation in the presence of these mercurials brought about a new uptake of Na^+ ion and water, and a loss of K^+ ion. The same response was found with $4–5 \times 10^{-4}$M-mercuric chloride. Chloride ion followed Na^+ ion changes.

3. Incubation of slices of kidney cortex for 30 min. in the presence of mercuric chloride or Esidrone at the above concentration brought about a loss of protein nitrogen into the medium, as compared with controls without mercurials.

4. Slices of kidney cortex, leached at 0° in 0·154M-sodium chloride containing 5×10^{-4}M-mercuric chloride or $1{\cdot}45 \times 10^{-3}$M-Esidrone or 10^{-4}M-*p*-chloromercuribenzoate, took up considerably more Na^+ ion and water than controls leached in sodium chloride alone. On further incubation at 25°, the decrease of Na^+ ion and water, and the uptake of K^+ ion, proceeded at the same rate as in controls. However, a new steady state was established in slices leached in the presence of mercurials, in that Na^+ ion and water came into equilibrium at higher levels, K^+ ion at lower levels, than in controls.

5. Measurements of fluxes of ^{24}Na showed that $1{\cdot}45 \times 10^{-3}$M-Esidrone increased the passive influx of Na^+ ion, leaving the active extrusion of Na^- ion unaffected.

6. The transport of Cl^- ion followed its own concentration gradient irrespective of whether mercurials were present or not.

7. Slices of brain cortex and myocardium took up more Na^+ ion and water on leaching at 0° in isotonic sodium chloride containing 5×10^{-4}M-mercuric chloride than controls leached without added mercuric chloride, while striated muscle (gracilis and diaphragm) and liver were not affected.

8. *p*-Quinone ($5 \times 10^{-4}–2 \times 10^{-3}$M) added during leaching in 0·154M-sodium chloride at 0° brought about an increased uptake of Na^+ ion and water. The effect of *p*-quinone was reversible on incubation at 25°.

9. The mechanism of diuretic action of mercurials is discussed. It is suggested that mercurials change the permeability of the basal membrane of renal tubular cells, thus increasing the passive movement of Na^+ ion, Cl^- ion and water back into cells, resulting in a decreased reabsorption of salt and water.

10. It is suggested that the state of SH groups of proteins in the membrane of renal tubular cells is a determinant of cell permeability to Na^+ ion and perhaps water.

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The Partial Purification and Properties of a Thiaminase from Bracken [*Pteridium aquilinum* (L.) Kuhn]

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Thiaminases were first recognized in certain species of fishes. Their discovery followed the demonstration that the disease in animal populations known as 'Chastek paralysis' was in fact a thiamine deficiency and developed when certain species of raw fishes were included in the ration of foxes. The substance in fish responsible for the destruction of the dietary thiamine was shown to be an enzyme by Sealock, Livermore & Evans (1943), and Krampitz & Woolley (1944). The relevant literature has been reviewed by Yudkin (1949) and Harris (1951).

The thiaminases are enzymes which catalyse the fission of the methylene–quaternary-nitrogen bond of thiamine. This usually occurs only in the presence of certain amines; the pyrimidine moiety is transferred to the amine according to the general equation

$$P{\cdot}CH_2{\cdot}T^+ + R{\cdot}NH_2 \rightarrow P{\cdot}CH_2{\cdot}NH{\cdot}R + T + H^+, \quad (1)$$

where P and T stand for the pyrimidine and thiazole components of thiamine respectively (Woolley, 1953; Fujita *et al.* 1952*c*; Sealock & Davis, 1949). Thiaminases catalysing the transfer reaction have been found in fishes, shellfish, bacteria and Pteridophytes (Harris, 1951; Fujita, 1954), but only with the bacterium *Bacillus aneurinolyticus* Kimura *et* Aoyama is there good evidence of the production of a thiaminase capable of catalysing the hydrolytic fission of thiamine (Fujita, Nose & Kuratani, 1954)

$$P{\cdot}CH_2{\cdot}T^+ + H_2O \rightarrow P{\cdot}CH_2OH + T + H^+. \quad (2)$$

Studies of plant thiaminase stem from the observation of Weswig, Freed & Haag (1946) that rats fed on a ration containing 40 % of air-dried bracken and adequate thiamine developed acute thiamine deficiency. Thomas & Walker (1949) confirmed this work and showed that bracken contained a thermolabile system capable of destroying thiamine at pH 4·5. The active system was extracted from the dried bracken leaf by Evans, Jones & Evans (1950), who concluded that it contained an enzyme and established that the thiazole component of thiamine was one of the products of the reaction. However, since Somogyi (1949) and Somogyi & Muralt (1949) claimed that the factor responsible for the inactivation of thiamine in fern or bracken extracts was thermostable (1 hr. at 100°) and that it passed a dialysing membrane, Evans & Jones (1952) repeated and extended their previous work. They found that over 90 % of the thiaminase activity of aqueous extracts of bracken was destroyed by heating for 15 min. at 100° and that dialysis separated the activity into thermolabile and thermostable fractions, both of which were necessary for enzyme action. The thermostable fraction could be replaced by the amines *m*-aminobenzoic acid and 5-aminosalicylic acid, which strongly suggested that bracken thiaminase catalyses the transfer reaction according to equation (1). The work of Fujita, Okamoto & Nose (1955) with the variety of bracken *P. aquilinum* var. *japonicum* supports this suggestion in that a thermolabile fraction which was activated seven- to eight-fold by certain amines was demonstrated in the extracts; when pyridine was used in the system fluorimetric evidence was obtained which suggested that the transfer reaction with the formation of *N*-(4-amino-2-methylpyrimidin-5-yl)methylpyridine (heteropyrithiamine) had taken place. However, in partial agreement with Somogyi (1949) and Somogyi & Muralt (1949), Fujita *et al.* (1955) found that a thermostable factor which was capable of destroying thiamine and which passed a dialysing membrane was also present in the extracts. The thermolabile factor of Fujita *et al.* (1955), unlike that of Evans & Jones (1952), did not lose activity on dialysis, which suggested that it might be able to catalyse the hydrolytic fission of thiamine.

The present work describes the partial purification and some properties of a thiaminase from bracken, which catalyses the transfer reaction according to equation (1). No evidence has been obtained that the preparations catalyse the hydrolytic fission of thiamine.

MATERIALS AND METHODS

Plant material. Bracken fronds were harvested locally in late June or July and air-dried at room temperature. The stems were discarded and the leaves were passed through a Christie–Norris Junior Laboratory Hammer Mill. The powder was stored at 0–4°. Other plant material was either glasshouse-grown in John Innes Compost or found growing wild.

Plant extracts. The plant material was ground with sand in a mortar with the addition of not more than an equal

weight of water and the pulp squeezed by hand through cotton cloth. The extract was centrifuged at 10 000 *g* for 5 min. and the supernatant dialysed at 0° overnight against distilled water.

Enzyme units. One unit of enzyme activity is that amount of enzyme which catalyses the formation of 1 μmole of heteropyrithiamine in 1 hr. under the standard conditions. The specific activity is the number of enzyme units/mg. of N of the enzyme preparation.

Estimation of thiamine. Thiamine was estimated after deproteinization of the reaction mixtures with HPO_3, either by the colorimetric method of Melnick & Field (1939), the intensity of the colour produced by the Prebluda–McCollum reagent being measured at 518 mμ, or by a modification of Gaudiano's (1954) method. This modification consisted of alteration in the strength of the reagents and omission of alcohol from the H_2O_2. Oxidation of the thiamine to thiochrome was accomplished by treating the samples with 1 ml. of ferricyanide reagent [8 ml. of 20% (w/v) NaOH + 2 ml. of 1% (w/v) $K_3Fe(CN)_6$, prepared immediately before use] for 1 min. and then adding 1 ml. of 0·05% H_2O_2. After standing for 20 min. with occasional shaking the absorption at 368 mμ was measured.

Nitrogen. This was determined by a micro-Kjeldahl method and the NH_3 measured by titration with HCl.

pH *measurements.* These were made with a glass electrode.

Spectrophotometry. A Unicam SP. 500 quartz spectrophotometer was used with 1 cm. glass or quartz cells.

Thiamine chloride hydrochloride. This was of B.P. quality from Roche Products Ltd.

Calcium phosphate gel. This was prepared by the method of Singer & Kearney (1950) and was aged at 0° for at least one month before use.

Buffers. Phosphate buffers were prepared from solutions of KH_2PO_4 or phosphoric acid and NaOH. Acetate and borate buffers were prepared from solutions of the acids and NaOH.

4-*Amino-5-hydroxymethyl-2-methylpyrimidine.* This was prepared by treating 4-amino-2-methyl-5-methylaminopyrimidine dihydrochloride with $NaNO_2$ according to the directions of Andersag & Westphal (1937).

4-*Amino-5-bromomethyl-2-methylpyrimidine hydrobromide.* This was prepared from the corresponding hydroxymethyl compound by treatment with HBr by the method of Cline, Williams & Finkelstein (1937).

N-(4-*Amino-2-methylpyrimidin-5-yl*)*methylpyridine bromide hydrobromide.* This was prepared by a method similar to that used by Wilson & Harris (1949) for the preparation of neopyrithiamine. 4-Amino-5-bromomethyl-2-methylpyrimidine hydrobromide (300 mg.) was dissolved in 12 ml. of *iso*propyl alcohol and 660 mg. of pyridine was added. The mixture was stood at 37° for 18 hr. The pale-yellow precipitate was washed with *iso*propyl alcohol and ether and dried *in vacuo*. Recrystallization from aqueous ethanol, and drying *in vacuo* over concentrated H_2SO_4, gave 147 mg. of white crystals, m.p. 260–261° (decomp.; uncorr.). Fujita, Nose, Uyeo & Koizumi (1952*b*) give 261° (decomp.) for material dried *in vacuo* at 100° (Found: N, 15·2; $C_{11}H_{14}N_4Br_2$ requires N, 15·45%).

Preparation of bracken thiaminase

Air-dried powdered bracken (40 g.) was stirred into 400 ml. of water containing 8 ml. of $CHCl_3$, and after stirring for 15 min. 5% (w/v) aq. NH_3 soln. was added to bring the pH to 8. Stirring was continued for a further 1·75 hr. in the cold room. The suspension was squeezed by hand through strong cotton cloth. The fibre was mixed with 200 ml. of cold water and again squeezed through the cloth. The combined extracts (about 500 ml.) contained 10 000–16 000 units of specific activity 40–50.

To the water extract at 0° solid $(NH_4)_2SO_4$ was added with stirring to a concentration of 650 g./l. After stirring for about 1 hr. the precipitate was collected by centrifuging at about 10 000 *g* for 10 min. and suspended in water (one-third of the original volume). The suspension was dialysed for 1 hr. against running tap water and then overnight against a large volume of distilled water at 0°. To the dialysed suspension sufficient 0·5 M-phosphate, pH 7·5, was added to bring the phosphate concentration to 0·01 M and it was then frozen for 24 hr. (Freezing considerably accelerated the filtration.) After thawing the suspension was filtered by suction through Hyflo Super-Cel; the pad was washed with a small volume of 0·01 M-phosphate, pH 7·5. A clear-brown filtrate, fraction A, containing 55–65% of the original activity, and with a specific activity of 100–120 was obtained.

To fraction A at 0°, solid $(NH_4)_2SO_4$ was added, with stirring, to a concentration of 500 g./l., and as soon as solution was complete the precipitate was centrifuged off at about 10 000 *g* for 5 min. The precipitate was suspended in water (about one-third of the volume of fraction A) and after brief dialysis against running tap water was dialysed against distilled water overnight at 0°. Most of the precipitate dissolved during the dialysis but a small amount of insoluble material was separated after dialysis by centrifuging at about 10 000 *g* for 5 min. The supernatant, fraction B, contained 72–84% of the activity of fraction A and had a specific activity of 450–480.

Further purification was accomplished by using calcium phosphate gel. The active material was most readily adsorbed by the gel at acid pH values and could be recovered in good yield by extraction with 0·1 M-phosphate, pH 7·5. The results were somewhat variable but the following method has given a useful purification on the three occasions it was tried.

Fraction B is mixed with the gel at 0° (about 450 mg. of gel/3000 units of enzyme) and the suspension adjusted to pH 5·5 with N-acetic acid. After standing for 20–30 min. at 0° the gel is centrifuged off and extracted 2–3 times with 0·1 M-phosphate, pH 7·5 (about 30 ml. total volume) at room temperature. The phosphate extract is then dialysed against water at 0° for 24–48 hr. In this way preparations of specific activity of 740, 960 and 1280 with 50–90% recovery of the activity have been obtained. The best preparation was obtained by successive treatment of fraction B with gel. A sample of fraction B of specific activity 460 containing 5930 units was treated with 380 mg. of gel as before and, after centrifuging, the supernatant was again treated with 170 mg. of gel. Dialysed phosphate eluates from the first gel contained 1490 units of specific activity 390, and from the second gel 3740 units of specific activity 3150. No appreciable loss of activity was observed with these purified fractions when they were stored for 3–4 weeks at −18°.

Measurement of thiaminase activity

For reasons which are discussed fully later it became desirable to develop a method of measuring thiaminase activity based on the formation of the pyrimidine–amine product. Preliminary experiments suggested that, of the

amines tested, pyridine was most active in the transfer reaction catalysed by bracken thiaminase. This reaction leads to the formation of 1 mole of heteropyrithiamine/mole of thiamine destroyed, and could be followed by measuring the rate of formation of heteropyrithiamine. A method of estimating small amounts of heteropyrithiamine in the presence of thiamine has therefore been worked out.

Estimation of heteropyrithiamine. Both thiamine and heteropyrithiamine are oxidized by alkaline ferricyanide with the formation of the fluorescent compounds thiochrome and 2-methylpyrichromine respectively (e.g. Barger, Bergel & Todd, 1935; Fujita, Nose, Ueda & Hasegawa, 1952*a*). In alkaline solution thiochrome has an absorption maximum at 368 mμ and 2-methylpyrichromine has a maximum at 386 mμ. This difference can be used for the determination of heteropyrithiamine in the presence of thiamine, but with mixtures in which the proportion of the latter is appreciable much of the absorption at 386 mμ is due to thiochrome, and attempts to develop a method based on the differing absorption spectra were finally abandoned in favour of that described below. The method is based on the fact that thiamine is unstable in alkali whereas heteropyrithiamine is unaffected. It was developed from the observation of Matsukawa & Yurugi (1954) that, when mixtures of thiamine and heteropyrithiamine were oxidized after treatment with strong alkali, the fluorescence of the solution then gave a measure of the heteropyrithiamine originally present.

In the present work it was found that solutions of thiamine incubated with NaOH and then treated with ferricyanide absorbed much less light at 386 mμ than those not previously incubated with NaOH (Fig. 1).

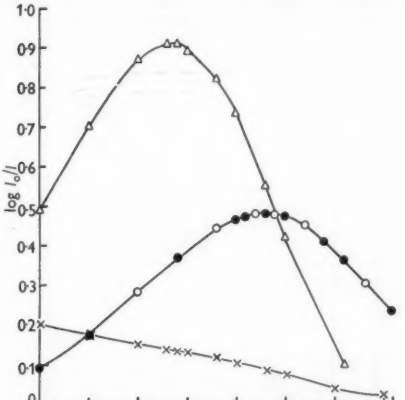

Fig. 1. Absorption spectra of the products obtained by oxidizing thiamine and heteropyrithiamine before and after treatment with NaOH as described in the text. △, Thiamine (reduced by one-half); ×, thiamine treated with NaOH before oxidation; ●, heteropyrithiamine; ○, heteropyrithiamine treated with NaOH before oxidation. Only a few of the experimental points are given.

Reaction mixtures, closely resembling those used in the enzyme assay (containing either 0·75 mg. of heteropyrithiamine bromide hydrobromide or 2 mg. of thiamine chloride hydrochloride, and 1 ml. of M-pyridine in a total volume of 10 ml., 0·025 M with respect to phosphate at pH 7·5) were made up and 2 ml. samples taken into 1 ml. of 20% (w/v) metaphosphoric acid. Samples (2 ml.) from these acid mixtures were then incubated for 1 hr. at 37° with 1 ml. of 20% (w/v) NaOH. Then 0·5 ml. of the ferricyanide reagent [4 ml. of 1% (w/v) $K_3Fe(CN)_6$ + 6 ml. of 20% (w/v) NaOH] was added and after 15 min. at room temperature the excess of ferricyanide was removed by the addition of 1 ml. of 0·05% H_2O_2. After standing for 20 min., with occasional shaking, the absorption spectrum was determined with a control mixture in the blank cell from which thiamine and heteropyrithiamine had been omitted. Also, 2 ml. samples of the acid mixtures were treated with alkaline ferricyanide without prior incubation with NaOH. With these the 1 ml. of NaOH was added at the same time as the ferricyanide reagent.

The results (Fig. 1) show that whereas the absorption of the heteropyrithiamine mixtures was unaffected by previous incubation with NaOH, that of the thiamine mixtures was considerably diminished. There was little difference, on a molar basis, in the absorption of the oxidation products of thiamine and heteropyrithiamine at 386 mμ, but when the oxidation was carried out after incubation with alkali the products derived from thiamine absorbed some 14–15 times less light at 386 mμ than those of heteropyrithiamine. There was no advantage in prolonging the time of incubation of the thiamine mixtures with NaOH since the absorption at 386 mμ after oxidation was not significantly diminished by a further incubation for 1 hr.

Results obtained with solutions of heteropyrithiamine bromide hydrobromide containing amounts of phosphate, pyridine and NaOH as described above showed that the maximum absorption at 386 mμ was reached within 5 min. of addition of the ferricyanide reagent. Increasing the time of oxidation to 30 min. did not increase the absorption.

The results of Fig. 2 were obtained with mixtures of thiamine, pyridine and phosphate similar to those described above except that the amount of heteropyrithiamine was varied. Samples were acidified with HPO_3, incubated with NaOH and treated with the ferricyanide reagent and H_2O_2 exactly as previously described, and the absorption at 386 mμ was measured. It can be seen that the relationship between the amounts of heteropyrithiamine taken and the absorption is linear and that the heteropyrithiamine–thiamine mixtures when corrected for the absorption of the thiamine give essentially the same values as those found for heteropyrithiamine alone. In this and other experiments it was found that heteropyrithiamine bromide hydrobromide in amounts of 0·25–2 mg./10 ml. of mixture containing 2 mg. of thiamine chloride hydrochloride could be measured with an error of less than 10%. With amounts of heteropyrithiamine bromide hydrobromide less than 0·25 mg./10 ml., where the absorption due to the heteropyrithiamine is of the same order as, or less than, that due to the thiamine, the error increases with decrease in heteropyrithiamine concentration. The major source of uncertainty under these conditions appears to be the variability of the absorption of the products of the alkali–ferricyanide-treated thiamine. Within a single experiment the optical density ($\log I_0/I$) varied by as much as $\pm 0{\cdot}008$, although such variation was

unusual, and in different experiments the optical density has varied from 0·07 to 0·12. In a number of experiments with heteropyrithiamine bromide hydrobromide over the range 0·25–2 mg./10 ml. of mixture, the variation of the optical density among different experiments was rarely greater than 5%.

Thiaminase assay. The conditions finally adopted for enzyme assay and referred to subsequently as the standard conditions were as follows.

At zero time enzyme was added to reaction mixtures containing 2 mg. (5·9 μmoles) of thiamine chloride hydrochloride, 1 ml. of 0·1 M-pyridine, 0·5 ml. of 0·5 M-phosphate and water to a total volume of 10 ml., at pH 7·5 and 37°. A 2 ml. sample was taken immediately into 1 ml. of 20% (w/v) HPO_3; after incubation for 0·5 hr. at 37°, a second sample was taken. After centrifuging, 2 ml. of the supernatant was incubated with 1 ml. of 20% (w/v) NaOH for 1 hr. at 37°. Oxidation with the ferricyanide reagent and H_2O_2 was carried out as described previously and 20 min. later the absorption at 386 mμ was measured, samples of a mixture from which thiamine and enzyme had been omitted, but treated with the other reagents, being used in the blank cell. The absorption does not alter appreciably in 1 hr. but a small decrease takes place on standing at room temperature overnight. Blank determinations with mixtures from which enzyme or thiamine had been omitted and with enzyme which had been heated for 15 min. before testing were run simultaneously with the experimental reaction mixtures. The increase in optical density in the blank determinations was always less than one-tenth of that found with the experimental mixtures. After subtracting the blank values the amount of heteropyrithiamine formed (μmoles) is calculated, a value for the molar extinction coefficient (ϵ) of 8000 for the oxidation product of heteropyrithiamine being used. Since the formation of heteropyrithiamine decreases the concentration of thiamine and hence its contribution to the absorption at 386 mμ, a correction allowing for this is calculated based on the absorption of the thiamine control mixture from which enzyme has been omitted. It was found that the absorption of such mixtures was proportional to the amount of thiamine present over the range 0·5–2 mg. of thiamine/10 ml. of mixture. It was also determined that small amounts (1 mg./10 ml. of mixture) of the thiazole moiety of thiamine, the other product of the enzyme action, did not contribute any significant absorption at 386 mμ after incubation with the NaOH and ferricyanide reagents.

Under the conditions for the enzyme assay described above, the rate of formation of heteropyrithiamine is directly proportional to the amount of enzyme used provided that the amount of heteropyrithiamine formed does not exceed 1·2 μmoles (Fig. 3). In practice it is preferable to work in the top part of the linear range with amounts of enzyme that will form about 0·7–1 μmole of heteropyrithiamine, since the estimation of heteropyrithiamine becomes increasingly subject to error with decrease in its concentration.

RESULTS

Effect of varying different factors on the activity of bracken thiaminase

Effect of pH. This was studied under the standard conditions except that different buffers were used. At pH values above 7 the final buffer concentration

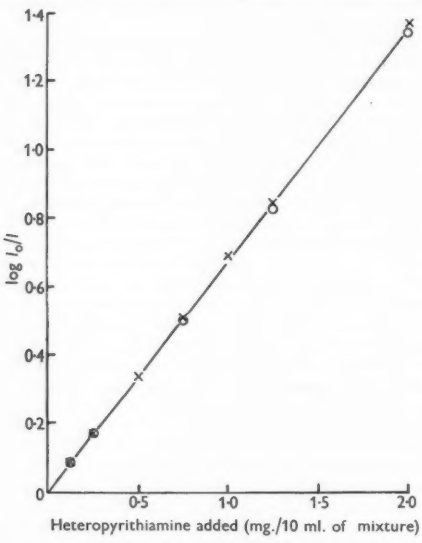

Fig. 2. Absorption of light at 386 mμ of heteropyrithiamine and heteropyrithiamine–thiamine mixtures after treatment with NaOH and ferricyanide. ○, Heteropyrithiamine alone; ×, heteropyrithiamine–thiamine mixtures corrected for the absorption of thiamine. Thiamine blank absorption was 0·102.

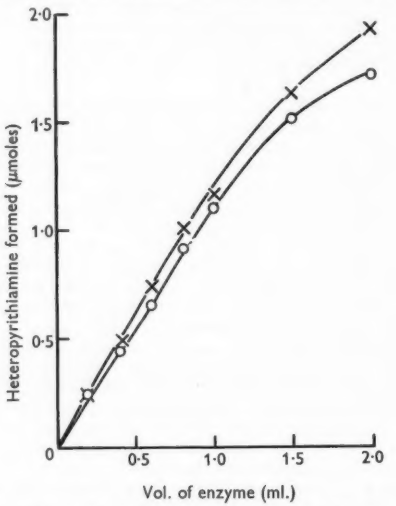

Fig. 3. Effect of enzyme concentration on the rate of formation of heteropyrithiamine. ○, Water extract of dried bracken leaf, specific activity 42; ×, bracken-thiaminase preparation (fraction B), specific activity 460.

was 0·01 M (phosphate and borate) and below pH 7 it was 0·01 M (phosphate and acetate). Where necessary the pyridine was adjusted to the appropriate pH value with N-HCl. The incubation period was 0·5 hr. and the enzyme used had specific activity 740. The difference between the initial and final pH of the reaction mixtures was within 0·2 pH unit. The results (Fig. 4) show that the optimum pH is in the neighbourhood of pH 7·5.

Effect of pyridine concentration. This was tested under the standard conditions, except that the amounts of pyridine were varied, an enzyme preparation of specific activity 740 being used. The results show (Fig. 5) that the activity increases with increase in pyridine concentration up to about 0·08 M and remains approximately constant over the range 0·08–0·3 M but decreases with further increase in pyridine concentration.

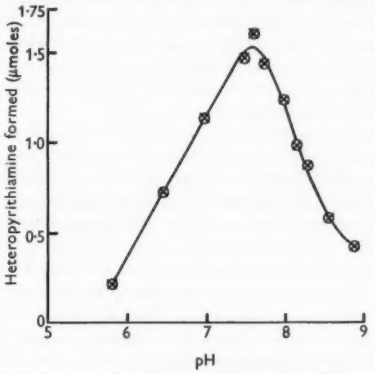

Fig. 4. Effect of pH on the rate of formation of heteropyrithiamine by a bracken-thiaminase preparation of specific activity 740.

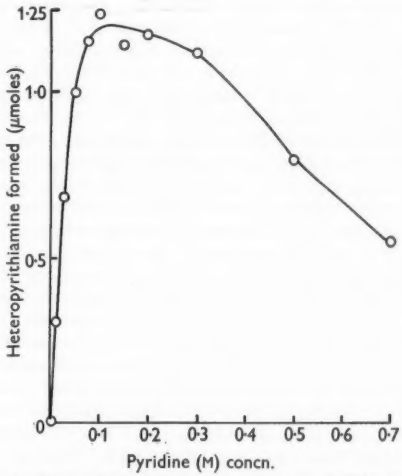

Fig. 5. Effect of varying the concentration of pyridine on the rate of formation of heteropyrithiamine by a bracken-thiaminase preparation of specific activity 740.

Effect of thiamine concentration. The method used for the determination of heteropyrithiamine does not lend itself to a study of the variation of enzyme activity with thiamine concentration. It is desirable to have at least 0·5 μmole of heteropyrithiamine formed in the reaction mixture, and this limits the lowest amount of thiamine which can be used. Further, if an amount of enzyme giving a reasonable activity at low thiamine concentration is employed with high thiamine concentration then the amount of thiamine remaining unchanged at the end of the experiment is high relative to the amount of heteropyrithiamine formed, and the thiamine blank absorption in the determination of heteropyrithiamine becomes unreasonably high. These difficulties could be partly overcome by using absorption cells with 4 cm. light path with thiamine concentrations below $1{\cdot}5 \times 10^{-4}$ M to increase the range of estimation of heteropyrithiamine, and, by using more enzyme, higher ranges of thiamine concentration could be investigated. In the present work the thiamine concentration was varied from 1·5 to $7{\cdot}4 \times 10^{-4}$ M (0·5–2·5 mg. of thiamine chloride hydrochloride/10 ml.) and the other conditions were standard. The results (Table 1) suggest that the maximum activity is reached at concentrations greater than $7{\cdot}4 \times 10^{-4}$ M.

Course of the reaction

Formation of heteropyrithiamine. Fig. 6 shows the rate of formation of heteropyrithiamine when either a crude enzyme (centrifuged water extract of dried bracken, specific activity 55) or a highly purified preparation (specific activity 3150) was

Table 1. *Effect of varying thiamine concentration on the rate of the thiaminase reaction*

Standard conditions were used (see Materials and Methods) and the thiamine concentration was varied. A partially purified bracken thiaminase of specific activity 3150 was used.

$10^4 \times$ Thiamine concn. (M)	Heteropyrithiamine formation (μmoles)
1·5	0·51
3·0	0·62
4·4	0·64
5·9	0·72
7·4	0·81

used. It can be seen that, provided sufficient enzyme is used, 90–95% of the thiamine added is finally present as heteropyrithiamine. With the complete reaction mixtures, only in those containing the lowest amount of water extract could the presence of thiamine be detected at the end of the experiment. In this mixture 5 μmoles of heteropyrithiamine had been formed and 0·3 μmole of thiamine remained out of the 5·9 μmoles of thiamine originally present. The blank values with enzyme, thiamine or pyridine omitted or with heated enzyme (15 min. at 100°) were very small, the largest being with heated enzyme, which is shown on Fig. 6. The results suggest that losses of thiamine by side reactions must be small.

Tests for hydrolytic fission of thiamine. With relatively large amounts of the purified preparations, tests were made to determine whether they contained thermolabile factors capable of catalysing the destruction of thiamine in the absence of added amine. Reaction mixtures consisted of 1 mg. of thiamine chloride hydrochloride, 1 ml. of phosphate–acetate buffer (0·25 M) and enzyme in a total volume of 10 ml. at pH 3, 4·5, 6 or 7·5. Two enzyme preparations were used: (1) 70 units of specific activity 740; (2) 154 units of specific activity 3150.

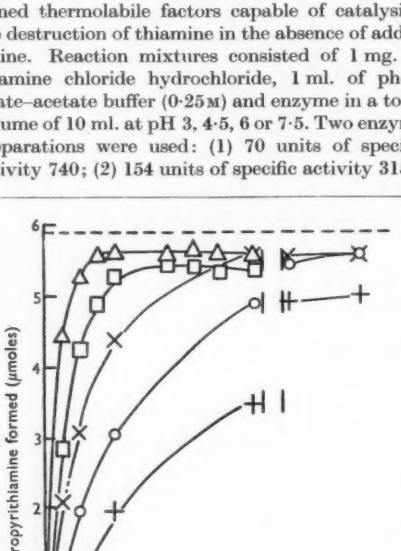

Fig. 6. Course of the reaction. Rate of formation of heteropyrithiamine with 0·05 ml. (+), 0·1 ml. (○) and 0·2 ml. (×) of centrifuged water extract of dried bracken leaf, and with bracken-thiaminase preparation of specific activity of 3150: 0·12 ml. (□), 0·24 ml. (△), and blank (●) with 0·2 ml. of water extract previously heated at 100°; ▲, blank with 0·24 ml. of thiaminase preparation previously heated at 100°. Other blanks with enzyme, thiamine or pyridine omitted were negligible.

The mixtures were incubated at 37° for 6 hr.; samples were taken into metaphosphoric acid at 0, 2 and 6 hr. After centrifuging, the thiamine in the supernatant was estimated by the modification of Gaudiano's method. Control mixtures with heated enzyme (15 min. at 100°), or from which thiamine or enzyme had been omitted, were also incubated along with the experimental mixtures. No evidence was obtained that either of the enzyme preparations contained a thermolabile factor capable of catalysing the destruction of thiamine. Preparation (1), 0·8 ml. (70 units), was mixed with 0·4 ml. of 0·001 M-$MnSO_4$ and set aside at room temperature for 15 min. The mixture was then tested as above but again with negative results. Also the enzyme–$MnSO_4$ mixture was diluted and tested for transfer activity with added pyridine, but the rate of formation of heteropyrithiamine was not affected by the treatment with $MnSO_4$.

Tests for synthesis of thiamine. Reaction mixtures containing 3 μmoles of 4-amino-5-hydroxymethyl-2-methylpyrimidine, 3 μmoles of 5-β-hydroxyethyl-4-methylthiazole, 1 ml. of phosphate–acetate buffer (0·25 M) and enzyme (70 units of specific activity 740) in a total volume of 10 ml. at pH 3, 4·5, 6 or 7·5 were incubated at 37° for 6 hr. After deproteinization with metaphosphoric acid, samples were tested for thiamine by the procedure of Melnick & Field (1939). No evidence of the formation of thiamine was obtained.

Other plants

Four species of higher plants and two species of ferns have been tested for transfer activity under the standard conditions. Extracts were made as described previously and tested immediately after dialysis. With the leaves of the ferns *Phyllitis scolopendrium* (L.) Newm. and *Dryopteris filix-mas* (L.) Schott, the specific activities of the dialysed extracts were 105 and 100 respectively as compared with 25 for a sample of fresh bracken leaf harvested and tested at the same time. By using larger amounts of enzyme and a longer incubation period the formation of heteropyrithiamine as a major product can be confirmed by determining the absorption spectra (350–415 mμ) of samples after treatment with NaOH and ferricyanide–H_2O_2. With both ferns the absorption spectra obtained in this way were almost identical with that found for heteropyrithiamine. Extracts of germinating soya bean (*Glycine soja* Sieb. & Zucc), parsley leaves [*Petroselinum crispum* (Mill.) Airy-Shaw], cabbage leaves (*Brassica oleracea* L.) and parsnip leaves (*Pastinaca sativa* L.) gave negative results even when large amounts (3 ml. of extract, 4–9 mg. of N) were used and incubation was prolonged to 3–5 hr.

DISCUSSION

The work referred to in the introduction of this paper shows that although it is generally agreed that extracts of bracken fronds are capable of destroying thiamine the nature of the factors involved have not yet been clearly identified. It seemed possible that there might be a thiaminase in bracken capable of catalysing both the hydrolytic fission of thiamine and the transfer reaction. Previously thiaminase activity has been followed by measuring the disappearance of thiamine, although evidence for the transfer reaction has been obtained by the isolation of the pyrimidine-amine product or by fluorimetric or chromatographic techniques. It seemed desirable therefore to develop a method by which the formation of the pyrimidine-amine product could be measured. The availability of such a method would make it possible to determine whether the activating effect of amines was wholly due to the transfer reaction or partly due to a stimulation of the hydrolytic fission of thiamine. Preliminary tests suggested that pyridine was highly active in the transfer reaction catalysed by bracken thiaminase, and a method of estimating the product (heteropyrithiamine) in the presence of thiamine was accordingly worked out. It has been shown by this method with both crude extracts of bracken and partially purified preparations that in the presence of excess of pyridine 90–95 % of the added thiamine is converted into heteropyrithiamine. This suggests that the destruction of thiamine by side reactions must be small. In the absence of pyridine, with amounts of enzyme capable of catalysing the destruction of 70–150 μmoles of thiamine/hr. in the transfer reaction, no evidence of a thermolabile factor capable of catalysing the destruction of thiamine was found. It has been reported that small amounts of Mn^{2+} ions increase the rate of destruction of thiamine by certain animal thiaminase preparations (Deolalkar & Sohonie, 1954; Reddy, Giri & Das, 1948). However, one of the partially purified preparations, after previous incubation with a small amount of Mn^{2+} ion, failed to destroy thiamine in the absence of pyridine. The transfer activity of the preparation with pyridine was not affected by the treatment with Mn^{2+} ion. No evidence of thiamine synthesis was obtained when one of the partially purified thiaminase preparations was incubated with 4-amino-5-hydroxymethyl-2-methylpyrimidine and 5-β-hydroxyethyl-4-methylthiazole. It must be concluded therefore that if the enzyme responsible for the transfer reaction is capable of catalysing the hydrolytic fission of thiamine its hydrolytic activity must be negligible in comparison with its transfer activity.

By analogy with the mechanism which is thought to hold for the other transferring enzymes the first step in the reaction would involve the formation of a pyrimidine–enzyme complex and 5-β-hydroxyethyl-4-methylthiazole (equation 3):

$$P{\cdot}CH_2{\cdot}T^+ + \text{enzyme} \rightarrow P{\cdot}CH_2{\cdot}\text{enzyme}^+ + T, \quad (3)$$

$$P{\cdot}CH_2{\cdot}\text{enzyme}^+ + C_5H_5N \rightarrow P{\cdot}CH_2{\cdot}N^+C_5H_5 + \text{enzyme}. \quad (4)$$

The pyrimidine–enzyme complex would then react with certain amines, e.g. pyridine (equation 4), but not to any appreciable extent with water. In this way it would differ from those enzymes which possess both hydrolytic and transferring activity.

The results of the present work are therefore in close agreement with those of Evans & Jones (1952), who found that both a thermolabile and a thermostable factor were necessary for enzyme action and that the thermostable fraction could be replaced by an aromatic amine.

The effect of pH and variation in pyridine and thiamine concentration on the transfer reaction catalysed by bracken thiaminase have been studied in order to find suitable conditions for the measurement of thiaminase activity. With these results a relatively simple method was worked out for the assay of bracken thiaminase. The method has proved useful in the preparation of partially purified thiaminase preparations, the best of which represented a 60-fold purification and a yield of about 30 %. With the method, thiaminase transfer activity was demonstrated in two other species of fern but no evidence of such activity was found in the few species of higher plants, including germinating soya beans, which were examined. Although Fujita (1954) found no thiaminase in germinating soya beans, Murata & Shiga (1952) have reported the presence of a thermolabile thiamine-destroying factor in extracts of such material. The results of the present work do not therefore conflict with those given by Fujita (1954), who examined a large number of Angiosperms and Pteridophytes for the presence of thiaminase. He found activity in all of the Pteridophytes tested but among the Angiosperms only in *Celosia crista*. Horses suffer from thiamine deficiency when the proportion of bracken in their diet is high (Roberts, Evans & Evans, 1949). It seems likely that the deficiency is brought about by destruction of the dietary thiamine by bracken thiaminase. It follows therefore that thiaminase is not likely to be present in the common plants of pastures.

Woolley (1953) has pointed out that many results on the activity and distribution of thiaminases are of doubtful value because the activity may have been limited by the amount of amines in the preparations tested. Kawamori (1955) failed

to detect thiaminase in the faeces of some human subjects unless pyridine was added to the reaction mixtures. Under the conditions used in the present work the activity of thiaminase increased with increase in pyridine concentration up to about 0·08 M. If the reaction mechanism outlined above is correct then the rate of formation of heteropyrithiamine would be expected to increase with increase in pyridine concentration. The fact that a maximum was obtained experimentally suggests that high pyridine concentrations may have undesirable effects on the enzyme or interfere with the reaction in some other way.

It has been shown (Fujita, 1954; Fujita & Tashiro, 1952; Fujita *et al.* 1952*a*, *c*) that pyridine increases the rate of destruction of thiamine by extracts and preparations from fishes, shellfish and bacteria, and fluorimetric or chromatographic evidence has been obtained (with the clam *Meretrix meretrix* L. proof was obtained by isolation of a derivative) of the formation of heteropyrithiamine. Although with some preparations, under the conditions used by these authors, amines other than pyridine were more active it appears probable that the present method of following transfer activity of bracken thiaminase may prove useful in studies of other thiaminases.

It is doubtful whether the enzyme approaches the maximum activity of which it is capable under the conditions used here for the assay. It has been shown that the enzyme is not saturated at the thiamine concentration used ($5{\cdot}9 \times 10^{-4}$ M), and further work may show that there are amines with a much higher activity than pyridine in the transfer reaction. From the results obtained, by assuming that the rate of thiaminase action at 17° is one-quarter of that found at 37° and that the dry weight of bracken frond is 20% of the fresh weight, the rate of metabolism of thiamine by thiaminase in the fresh leaf could be of the order of 4000 μg./g. of leaf/hr. at 17°. Watanabe (1952) finds that bracken contains 0·66 μg. of thiamine/g. fresh wt. It is by no means certain that thiamine is the substrate of thiaminase *in vivo*. If it is, then thiaminase would catalyse the synthesis of thiamine analogues having an amine other than 5-β-hydroxyethyl-4-methylthiazole attached to the methylene bridge. Such compounds are as yet unknown in biological material. Thiaminase will act on substrates having structural features resembling thiamine (Fujita, 1954), and it appears probable that *in vivo* it may play some role in the synthesis of compounds other than thiamine analogues. Woolley (1951) has provided some experimental support for this by demonstrating that carp thiaminase catalyses the transfer reaction between the pteridine analogue of thiamine [3-(2-amino-4-hydroxypteridin-6-ylmethyl)-4-methyl-5-β-hydroxyethylthiazolium bromide] and *p*-aminobenzoic acid or *p*-aminobenzoylglutamic acid, with the formation of pteroic acid or pteroylglutamic acid, although the yields were extremely small. Woolley does not consider that this is the mode of biosynthesis of these compounds. The results do, however, indicate the potentialities of thiaminase as a synthesizing enzyme. Thiaminase apparently has a limited and erratic distribution (Harris, 1951; Fujita, 1954). Among the fishes, for example, it occurs only in some fresh-water and salt-water species. It could be that thiaminase has some special role in those organisms in which it occurs. Alternatively, *in vivo* it may catalyse a reaction common to most organisms and only show activity with thiamine *in vitro* in certain cases.

With certain amines the transfer reaction (equations 3 and 4) is reversible (Fujita *et al.* 1952*b*). Therefore the possibility exists that thiaminase takes part in the synthesis of thiamine by catalysing the exchange of 5-β-hydroxyethyl-4-methylthiazole with a pyrimidine–amine precursor. Such a role for thiaminase would appear to presuppose different pathways of thiamine synthesis in, for example, different but closely related fishes.

SUMMARY

1. A method for the estimation of small amounts of heteropyrithiamine [*N*-(4-amino-2-methylpyrimidin-5-yl)methylpyridine] in the presence of thiamine is described. It depends on the destruction of thiamine by incubation with strong alkali and subsequent oxidation of heteropyrithiamine to 2-methylpyrichromine by ferricyanide. The 2-methylpyrichromine is estimated spectrophotometrically at 386 mμ.

2. With the method it is shown that bracken thiaminase catalyses a transfer reaction between thiamine and pyridine with the formation of heteropyrithiamine. In the presence of excess of pyridine the amount of heteropyrithiamine formed accounts for 90–95% of the thiamine added.

3. A relatively simple method for the measurement of bracken-thiaminase activity is described.

4. By fractionation with ammonium sulphate and calcium phosphate gel, concentrated partially purified thiaminase preparations have been made from water extracts of dried bracken leaves. The best preparation was obtained in 30% yield and represented a purification of about 60-fold. It catalysed the formation of 3150 μmoles of heteropyrithiamine/hr./mg. of N at 37°.

5. With the concentrated partially purified preparations no evidence was obtained of the presence of a thermolabile factor capable of catalysing the hydrolytic fission of thiamine.

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Tissue Components of the Domestic Fowl

2. BLOOD UREA*

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The domestic fowl, besides being of great economic importance, is often used as an experimental animal. Pathological abnormalities of various organs which arise from time to time, even under the best husbandry, are rarely detectable except at post-mortem examination. The investigation of avian urine for pathological constituents is at present impracticable; blood examination and tissue biopsy afford the only approaches to diagnosis by chemical means. The flock of brown leghorns at the Poultry Research Centre is carefully isolated and is not subject to contagion from outside; nevertheless, organ dysfunctions, e.g. of the kidney, are sometimes encountered. The possibility that some aspect of blood analysis might lead to early detection of kidney dysfunction is under investigation.

The literature from 1923 to 1952 contains conflicting reports on blood urea of presumably healthy fowls; the figures quoted cover a remarkable range despite the view generally accepted by biologists that urea is found only in low concentrations in normal avian tissues (cf. Clementi, 1914, 1915, 1918, 1922, 1932; Hunter & Dauphinée, 1925; Pitts & Korr, 1938; Howell, 1939*a*).

In the literature results have been expressed in two ways: as 'mg. of urea' and as 'mg. of urea nitrogen'. As the former is quantitatively more than twice the latter, this is a matter of some moment, since papers exist where both modes of expression are used indiscriminately to cover the same or similar sets of values (e.g. Hogan, Shrewsbury & Kempster, 1929; Herrmann, 1946*a*, *b*). The collated summaries of Shimer (1937), Howell (1939*a*) and Sturkie (1954) contain errors arising from confusion of the two values.

This paper describes an examination of the blood-

* Part 1: Bell (1957).

urea content of healthy fowls and of a few birds proved by post-mortem examination to have been suffering from early pathological lesions of the kidney glomeruli. The Conway apparatus was used with urease which had been tested against possible interfering blood components.

METHOD

Both the healthy and sick birds were allowed free access to water and to the normal diet of the Poultry Research Centre (Bell, 1957). Blood was taken from the wing vein into citrate (2 mg./ml. of blood).

In blank determinations, the volatile base ('ammonia') released in appreciable amounts from the blood had, of course, to be measured. This 'ammonia' is of no significance *in vivo* since Conway & Cooke (1939) have shown that, in birds as well as in other terrestrial animals, it is impossible to detect ammonia in freshly shed blood. It originates in birds by enzymic deamination of adenine, guanine, guanosine, cytidine and cytosine after the blood is shed.

Urea determinations by diffusion (devised by Mr T. P. Bird)

The procedure of Conway (1947) for mammalian blood had to be modified only with respect to permitting titration of the ammonia formed by urease from the small amounts of urea in 1 ml. of avian blood. With ordinary microburettes this was found possible by acidimetry, iodate–iodide with thiosulphate being used (cf. Bang, 1916).

The iodate–iodide solution was prepared before each batch of titrations by mixing equal volumes of 2% (w/v) KIO_3 and 40% (w/v) KI. The concentration of KI is fairly critical; if too much is present it interferes with the blue colour of the starch indicator, which becomes reddish, and a satisfactory end-point cannot be obtained. If insufficient KI is present iodine is lost by volatilization.

(*a*) *Ammonia recovery.* Ammonium sulphate solution (1 ml.; N content, 0·0159 mg.) was placed in the annular space, and 0·5 ml. of 0·01 N-H_2SO_4 in the centre compartment, of a Conway no. 1 unit. Saturated K_2CO_3 solution (1 ml.) was added to the annular compartment and, after sealing the lid, the whole was incubated at 38° for 2 hr. Blanks with water in place of $(NH_4)_2SO_4$ were run simultaneously. The acid in the centre compartments was titrated iodometrically; 0·25 ml. of iodate–iodide solution was added to the centre compartment and mixed with a fine rod. After 1 min., to allow the reaction to come to completion, the iodine was titrated with 2 mN-$Na_2S_2O_3$, with one drop of 1% starch solution as indicator. Triplicate experiments gave a recovery of 100%.

(*b*) *Urea-recovery tests.* Urea solution (1 ml., 0·0443 mg.) was placed in the annular space along with 1 ml. of M/15 phosphate buffer (pH 7·38) and a small knife-point of urease powder (L. Light and Co.); 0·5 ml. of 0·01 N-H_2SO_4 was placed in the centre compartment and, after sealing, the whole was incubated at 38° for 15 min. Saturated K_2CO_3 solution (1 ml.) was added to the fluid in the annular compartment and, after mixing, incubation was continued for 2 hr. The residual acid in the centre compartment was then determined as in (*a*) above (average titration, 1·72 ml. of 2 mN-$Na_2S_2O_3$), the blank experiment having water in place of urea solution (titration 2·48 ml.). The difference of 0·76 ml. is equivalent to 0·0456 mg. of urea, i.e. 103% recovery.

Under the conditions of (*b*) above no measurable volatile base was yielded by the enzyme alone, the buffer alone and the buffered enzyme with allantoin and with L-arginine.

(*c*) *The method applied to blood.* The annular space was charged with 1 ml. of blood, 1 ml. of buffer and a small knife-point of urease; 0·5 ml. of 0·01 N-H_2SO_4 was placed in the centre compartment. The subsequent procedure was as for (*a*) above. The blank contained 1 ml. of water instead of blood.

RESULTS

The values for the blood urea of presumably healthy birds are given in Table 1. Among the older hens no differences were found between the laying and non-laying conditions. Also recorded are measurements made on birds found to have early glomerulonephritis (Table 2).

DISCUSSION

A summary of previous results and methods, and of the present experiments, is given in Table 3. Wide variations in the urea levels, and their ranges, cannot be attributed solely to the use of particular methods. It is accepted that urease will quantitatively, and specifically, decompose urea, other ammonia-forming enzymes and their substrates being absent (Howell, 1939*b*; Damodaran & Sivaramakrishnan, 1946); gross errors must therefore be sought in some point of technique other than the initial enzymic treatment. In the methods of Folin & Wu (1919), Karr (1924), Leiboff & Kahn (1929) and Kibrick & Skupp (1950) the ammonia formed is measured by nesslerization, which Folin & Wu, however, stated to be unsatisfactory in the presence of either amino acids or peptones. Referring to urea nitrogen, Folin & Wu also say 'whether one finds . . . 3 instead of 2 mg. in 100 c.c. of chicken

Table 1. *Blood urea of normal fowls*

			Urea (mg./100 ml.)			Urea N (mg./100 ml.)		
Sex	Age	No.	Range	Mean	S.D.	Range	Mean	S.D.
Female	1 month*	6	1·4–3·4	2·38	0·76	0·65–1·59	1·11	0·37
Male	1 month*	8	1·4–4·6	2·73	1·00	0·65–2·15	1·28	0·47
Female	1–5 years	14	1·7–3·9	2·69	0·79	0·79–1·82	1·23	0·37
Overall total		28	1·4–4·6	2·64	0·79	0·65–2·15	1·23	0·37

* Hsu & Combs (1952) reported a range of urea nitrogen of 3·8–18·5, mean 9·8, mg./100 ml. of blood for birds in this age group.

Table 2. *Blood urea of hens with defective glomeruli but normal tubules*

Bird no.	Age (years)	Urea (mg./100 ml.)	Urea N (mg./100 ml.)	Pathology*
1	4	2·4	1·12	Atherosclerosis. Proliferative changes in glomeruli
2	4	2·8	1·30	Metastasizing adenocarcinoma of oviduct. Low-grade glomerulosclerosis
3	3	3·8	1·77	As for 1
4	3	2·9	1·35	As for 1
5	2	1·9	0·89	No vascular lesions. Low-grade proliferative glomerulonephritis
6	2	3·5	1·63	As for 5
7	2	1·9	0·89	No vascular lesions. Acute diffuse proliferative glomerulonephritis
	Average	2·7	1·28	

* Reports kindly provided by Dr W. G. Siller.

Table 3. *Published values for urea (all expressed as urea nitrogen) in fowl blood*

Use of Nessler's reagent is indicated by (Ne).

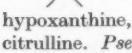
Authors	Method	Urea N (mg./100 ml.) Highest	Lowest	Average
Scheunert & Pelchrizm (1923)		16·9	7·3	(Ne) 12·1
Hayden (1929)		3·6	1·2	(Ne) 2·5
Hogan *et al.* (1929)	Folin & Wu (1919)	5·4	2·3	(Ne) 3·8
Hayden & Brunett (1930)		7·5	5·1	(Ne) 6·6
Hayden & Sampson (1931)		4·5	2·1	(Ne) 3·3
Gonzago (1935)	Karr (1924)	2·7	2·0	(Ne) 1·3
Heller & Pursell (1937)	Folin & Svedberg (1930)	5·0	2·2	3·6
Dyer & Roe (1934)		2·5	1·0	(Ne) 1·5
Herrmann (1946*a*)	Leiboff & Kahn (1929)	—	—	(Ne) 8·9
Herrmann (1946*b*)		—	—	(Ne) 19·0
Hsu & Combs (1952)	Kibrick & Skupp (1950)	18·5	3·8	(Ne) 9·8
Pitts & Korr (1938)	Van Slyke (1927)	1·7	0·0	—
Howell (1939*a*)	Howell (1939*b*)	—	—	0·7
Present work	Conway (1947), modified	2·2	0·7	1·2

blood is as yet of comparatively small consequence'. Peters & Van Slyke (1932), referring to nesslerization of deproteinized blood filtrates, state: 'Other constituents of blood filtrates apparently increase the intensity of the colour produced.' Connerty, Briggs & Eaton (1955) have emphasized that the colour produced by direct nesslerization of protein-free blood filtrates is not stable and that the optical density increases steadily until turbidity is produced. This point was in fact made by Scheunert & Pelchrizm (1923), who obtained very high values for the apparent urea (Table 3). Turbidity is especially likely to develop in warm weather (Levey & Reid, 1952). Appreciable quantities of amino acids are present in fowl-blood filtrates (Dunn, 1949; Salander & Fisher, 1956; Bell & Gross, unpublished work). Interference by organic vapours is another comparatively unrecognized hazard in nesslerization.

The method of Folin & Svedberg (1930), which involved distillation and titration, should be accurate, but it gave high results in the hands of Heller & Pursell (1937) and Shimer (1937); the last-named also used Karr's (1924) method without differentiating the results of the two methods.

Direct chemical hydrolyses (Leiboff & Kahn, 1929; Kibrick & Skupp, 1950; Owings & Mandel, 1951) would also include ammonia from amides other than urea; the amide content of avian blood is not known.

The results of the present work are only slightly higher than those obtained previously by urease–aeration (Howell, 1939*a*, *b*) and urease–manometry (Pitts & Korr, 1938); nesslerization has given comparable results only at the hands of Dyer & Roe (1934) and Gonzago (1935). Clementi (1932), by the xanthydrol method checked by urease, also found values within the range 0–2 mg./100 ml. The present author is convinced that this is in fact the range in the healthy domestic fowl and that much higher values are due to technical faults. The blood urea may be raised in some diseases, as Howell (1939*a*) has found. Pitts & Korr (1938) conclude that urea is excreted solely by the glomerulus, about 25 % being re-absorbed by the tubules, but no elevation has been found in the pathological cases examined by the writer, which comprised birds showing limited proliferative and sclerotic changes in the glomeruli (Table 2).

The origin of avian-blood urea is not yet established. Clementi (1914, 1915, 1918, 1922) and Hunter & Dauphinée (1925) were unable to show the presence of arginase in avian liver; kidney, however, contains the enzyme. The latter authors suggest that the role of kidney arginase may be to produce the ornithine required to synthesize ornithuric acid. It is hoped to examine this possibility. Whether avian kidney, *in vivo*, does in fact generate the blood urea is not known; mammalian blood is free from ammonia, yet mammalian kidney hydrolyses glutamine to give NH_4^+ ion, which is apparently entirely lost to the urine. Avian urine also contains NH_4^+ ion. Since mammalian and avian metabolic systems need not necessarily follow the same pathways, sources of avian urea alternative to arginine may exist. Krebs & Eggleston (1939) have studied a *Corynebacterium* which can convert into urea the grouping  contained in creatine, creatinine, hypoxanthine, uric acid, allantoin, arginine and citrulline. *Pseudomonas* spp. and a mould possess similar capacities (cf. Nimmo-Smith & Appleyard, 1956; Appleyard & Woods, 1956). All these substrates are known constituents of fowl blood, except citrullin and allantoin. Circumstantial evidence for the presence of the latter in fowl urine has been offered by Coulson & Hughes (1930). Enzymic mechanisms first encountered in micro-organisms are often subsequently discovered to exist among the metazoa.

SUMMARY

1. Measured by urease in the Conway apparatus, the urea-nitrogen content of healthy fowl blood averages about 1·2 mg./100 ml., in reasonable agreement with some, but not all, previous investigations. Very high values which have been reported in the literature, e.g. 12–19 mg. of urea nitrogen/100 ml. of blood, are believed to arise from technical failures.

2. No elevation in blood urea was found in a number of birds which had been suffering from early glomerulonephritis.

3. The origin of avian blood urea is briefly considered.

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The Effect of Nitrogen Mustards on the Incorporation of Amino Acids into Protein by *Staphylococcus aureus*

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Nitrogen mustards have been used clinically with some success in the treatment of malignant lymphadenopathies and the chronic leukaemias (see, for instance, Galton, Israels, Nabarro & Till, 1955), and they are also effective in inhibiting the growth of some solid tumours in experimental animals. Their biochemical mode of action remains obscure, although many studies *in vitro* have shown that they readily enter into chemical combination with nucleic acids and proteins under very mild conditions (reviews by Alexander, 1954 and Ross, 1952; also Cohn & Crathorn, unpublished work). More recently, Cohn (unpublished work) has found that *p*-di-(2-chloroethyl)amino-DL-phenylalanine becomes firmly bound to the proteins of liver and other tissues when the compound is injected interperitoneally into rats.

The present investigation represents an attempt to determine the extent to which the nitrogen mustards exert their effects on the direct incorporation of amino acids into proteins. A bacterial system was selected because of its simplicity. The use of *Staphylococcus aureus* offered a further advantage: only in this organism have two modes of amino acid assimilation been demonstrated, one associated with net protein synthesis and the other with an 'exchange' reaction between free amino acids and the amino acid residues within the preformed protein molecules (Gale & Folkes, 1953*a*).

METHODS

Organism. The organism used throughout this work was *Staphylococcus aureus* strain Duncan (Gale & Folkes, 1953*b*), which was kindly supplied by Dr K. McQuillen. It was maintained by frequent subculture on solid peptone–agar slopes. Larger batches for experimental purposes were obtained by growing the organism on a liquid medium of the following composition (%): glucose, 1; Marmite, 0·5; peptone, 1; NH_4Cl, 0·2; Na_2HPO_4, 0·6; KH_2PO_4, 0·3; NaCl, 0·3; $Na_2SO_4,10H_2O$, 0·025; $MgCl_2,6H_2O$, 0·0085 (all w/v). At 37° on this medium the yield of cells was approx. 1 mg. dry wt./ml. when grown for 16 hr. in Roux bottles.

Preparation of suspensions and conditions for study of uptake of ^{14}C*-labelled amino acids.* The bacteria were harvested after overnight growth and washed with water. Suspension densities were determined from the absorption at 420 mμ on a Unicam spectrophotometer (Model SP. 600) previously calibrated against the organism used. Thick suspensions of the organism (50 mg. dry wt./ml.) were then prepared in 0·05 M-phosphate buffer (prepared from KH_2PO_4, brought to pH 7 with NaOH) containing 1% (w/v) glucose, and 5 ml. samples were pipetted into each flask; the nitrogen mustards to be studied were added to the individual flasks at this stage. After aerobic incubation, while shaking at 37° for 0·5 hr., the ^{14}C-labelled amino acid was added, and the incubation continued for a further period of 90 min. In this way the labelled amino acid was taken up under exchange conditions. In experiments where an investigation was made of the effects of nitrogen mustards on uptake under conditions of net protein synthesis the bacteria were collected and transferred to an inorganic salts–glucose medium containing a complete mixture of amino acids. The labelled amino acid was then added and the incubation continued as in the experiments carried out under exchange conditions. The incubation was stopped by adding aq. trichloroacetic acid to a final concentration of 5% (w/v) and cooling to 2°. The precipitated proteins were collected and extracted first with water–ethanol (1:3) at 37° for 18 hr. and then with water–ethanol–ether (1:3:4) at 37° for 1 hr. The nucleic acids were then removed by two treatments at 95° with 5% (w/v) trichloroacetic acid. The proteins were finally washed twice with acetone and dried at 70°.

Toxicity tests. The incubations of buffered bacterial suspensions with nitrogen mustards were carried out at 37° for periods similar to those used in the examination of the uptake of amino acids by the cells. Viable cell counts were then made on peptone–agar plates after serial dilutions of the bacterial suspensions with sterile water. These dilutions had reduced the viable counts to the order of a few hundred/plate in control suspensions untreated with nitrogen mustard.

Assays of radioactivity. These were carried out on 'infinitely thick' samples of protein plated on 1 cm.^{2} polythene disks (Popják, 1950), an EHM 2S end-window Geiger–Müller counting tube supplied by G.E.C., London, being used in conjunction with a type 100C counting unit manufactured by Panax Equipment Ltd., Mitcham, Surrey. The counter was housed in a lead castle manufactured by E.R.D. Ltd., Slough, Bucks. Under these conditions a ^{14}C-labelled protein sample of specific activity 1 μc/g. would give an observed counting rate of about 600 counts/min. when plated at 30 mg./cm.^{2} (i.e. a counting efficiency of 1·2%).

Materials. [1-^{14}C]Glycine was supplied by the Radiochemical Centre, Amersham, Bucks. DL-[3-^{14}C]Phenylalanine was synthesized in these laboratories by Dr V. C. E. Burnop.

Details of the syntheses of the nitrogen mustards used are to be found in the recent literature (Everett, Roberts & Ross, 1953; Bergel & Stock, 1954; Davis, Roberts & Ross, 1955; Bergel, Everett, Roberts & Ross, 1955*b*).

Tumour-inhibition tests. (Personal communication from Professor A. Haddow.) Tumour-growth-inhibition tests by nitrogen mustards were carried out on the transplanted Walker rat carcinoma (Badger, Elson, Haddow, Hewett & Robinson, 1942; Haddow, Harris, Kon & Roe, 1948). Most of the results quoted here have been briefly reported elsewhere (Everett *et al.* 1953; Davis *et al.* 1955; Bergel *et al.* 1955*b*).

RESULTS

The initial experiments were carried out with three aliphatic nitrogen mustards: dimethyl-(2-chloroethyl)amine (M1), methyldi-(2-chloroethyl)amine (M2) and tri-(2-chloroethyl)amine (M3) (see Table 1). M2 is widely used in the treatment of leukaemia and allied diseases, it being often preferred to M3, which is rather more active but also more toxic than M2 (Galton, private communication). It was found (Table 1) that the inhibition of the amino acid-exchange reaction in our system paralleled the therapeutic efficiency of the compounds, the inhibition of incorporation being greater than would be explained by the reduction in viability of the organism. In a further experiment, the uptake of amino acids by the M3-poisoned system was studied under conditions where amino acid exchange could be compared with the uptake of amino acids under conditions otherwise favourable for net protein synthesis. The inhibitions of amino acid uptake were the same in the two cases, contrary to a statement made in an initial report of this work (Crathorn & Hunter, 1956). It therefore appears that with these substances the inhibition of the exchange reaction runs parallel with the inhibition of overall protein synthesis.

In experiments with other nitrogen mustards, 'exchange' conditions were normally used, as it was possible to simplify the procedures by using thick suspensions of bacteria in relatively small volumes of buffered solutions.

The series of aromatic nitrogen mustards bearing aliphatic acid side chains was next studied, as in this series there is an interesting variation in the activities of different compounds on the Walker rat carcinoma; this series also contains one of the compounds (M5) that has been used therapeutically. Here a very close correspondence between the activities on the Walker tumour and the amino acid-exchange system was obtained (Table 2). Unfortunately, a supply of the other inactive member with normal alipatic side chains, namely *p*-di-(2-

Table 1. *Comparison of the effectiveness of aliphatic nitrogen mustards in cancer chemotherapy with their ability to reduce the extent of the amino acid uptake in* Staphylococcus aureus *and their toxicity towards this organism*

Nitrogen mustards were used in final concentrations of 6·4 mM. DL-[3-^{14}C]Phenylalanine (specific activity 12 μc/mg.) was added to give a final concentration of 0·32 μc/ml. Under the conditions described in the text, the control cultures incorporated ^{14}C to the extent of approx. 1 μc/g. of protein.

Reference used in text.	Chemical formula of the nitrogen mustards	Effectiveness in cancer chemotherapy	Inhibition of the exchange reaction with phenylalanine (%)	Reduction in viability of the organism under similar conditions (%)
M1	$(CH_3)_2N{\cdot}CH_2{\cdot}CH_2Cl$	–	0	0
M2	$H_3C{\cdot}N(CH_2{\cdot}CH_2Cl)_2$	+ +	75	50
M3	$N(CH_2{\cdot}CH_2Cl)_3$	+ + +	98	50

Table 2. *Comparison of the inhibitory effect of acidic aromatic nitrogen mustards on the Walker rat carcinoma with their ability to reduce the extent of amino acid uptake in* Staphylococcus aureus

Nitrogen mustards were added to give a final concentration of 3·3 mM. DL-[3-^{14}C]Phenylalanine (specific activity 12 μc/mg.) was used in a final concentration of 0·32 μc/ml. Under the conditions described in the text, the control cultures incorporated ^{14}C to the extent of approx. 1 μc/g. of protein.

Reference used in text	R, in chemical formula of the nitrogen mustard $(Cl{\cdot}CH_2{\cdot}CH_2)_2N$-*p*-$C_6H_4{\cdot}R$	Assessment of inhibition of Walker carcinoma	Inhibition of the exchange reaction with phenylalanine (%)
M4	$\cdot CH_2{\cdot}CO_2H$	+ +	95
M5	$\cdot[CH_2]_3{\cdot}CO_2H$	+ + +	97
M6	$\cdot[CH_2]_4{\cdot}CO_2H$	–*	5
M7	$\cdot O{\cdot}[CH_2]_3{\cdot}CO_2H$	+	44
M8	$\cdot O{\cdot}[CH_2]_4{\cdot}CO_2H$	+	49

* Results of tests were variable. Occasionally found: +.

chloroethyl)aminophenylpropionic acid, was not available. Neither the substituted phenylbutyric acid (M5) active against the Walker rat carcinoma nor the inactive phenylvaleric acid analogue (M6) was at all toxic to the bacteria under our conditions and time of exposure, so that here the inhibition of amino acid uptake was even more clearly divorced from the direct toxic effects than with the aliphatic series of nitrogen mustards.

The nitrogen mustard analogue derived from phenylalanine was also examined (Table 3). Here the compound has been resolved (Bergel, Burnop & Stock, 1955*a*), and the different optical isomers exhibit different activities against the Walker rat carcinoma. It was found that amino acid uptake in our system was most effectively inhibited by the L-isomer (M11). After hydrolysing the compound in 0·05M-phosphate buffer, pH 7 (KH_2PO_4–NaOH mixture), for 2 days at 37°, no inhibition of the amino acid exchange is observed. It is thus clear that the inhibition is essentially a property of the chloro-compound. It must be stated that considerable variation was found in the percentage inhibition produced by different batches of this nitrogen mustard, and the figures given in Table 3 are representative of the relative values which have always been found.

Finally, a group of aromatic nitrogen mustards bearing amine side chains was tested. These substances have been found to be, in general, less active against the Walker rat carcinoma than those described above, and not many of them showed any appreciable activity in our staphylococcal system. Certain discrepancies are, however, evident from an examination of Table 4, and these are discussed in the next section.

Although most of the measurements of amino acid uptake were made with DL-[3-^{14}C]phenylalanine as the labelled amino acid, on occasion this was replaced by [1-^{14}C]glycine with similar results.

DISCUSSION

Confining our attention, initially, to the results obtained on the bacterial system, we may consider what can be deduced about the primary biochemical action of these mustards.

It might be thought that a significant proportion of the inhibitory effect produced by certain mustards could be caused by chemical interaction of the

Table 3. *Comparison of the effectiveness of optical isomers of* p-*di*-(2-*chloroethyl*)*aminophenylalanine and the hydrolysed* L-*isomer in inhibiting the Walker rat carcinoma with their ability to reduce the extent of amino acid uptake into protein by* Staphylococcus aureus

Nitrogen mustards were added to give a final concentration of 3·3 mM. DL-[3-^{14}C]Phenylalanine (specific activity 12 μc/mg.) was used in a final concentration of 0·32 μc/ml. Under the conditions described in the text, the control cultures incorporated ^{14}C to the extent of approx. 1 μc/g. of protein.

Reference used in text	Isomer of 'phenylalanine nitrogen mustard'	Assessment of inhibition of Walker rat carcinoma	Inhibition of the exchange reaction with phenylalanine (%)
M9	DL	+ +	56
M10	D	+	28
M11	L	+ + +	75
M12	L (after hydrolysis)	·	10

Table 4. *Comparison of the inhibitory effect of basic aromatic nitrogen mustards on the Walker rat carcinoma with their ability to reduce the extent of the amino acid uptake by* Staphylococcus aureus

Nitrogen mustards were added to give a final concentration of 3·3 mM. DL-[3-^{14}C]Phenylalanine (specific activity 12 μc/mg.) was used in a final concentration of 0·32 μc/ml. Under the conditions described in the text, the control cultures incorporated ^{14}C to the extent of approx. 1 μc/g. of protein.

Reference used in text	R, in chemical formula of the nitrogen mustard $(Cl{\cdot}CH_2{\cdot}CH_2)_2N$-*p*-$C_6H_4{\cdot}R$	Assessment of inhibition of Walker rat carcinoma	Inhibition of the exchange reaction with phenylalanine (%)
M13	$\cdot NH_2$,HCl	+(?)	9
M14	$\cdot CH_2{\cdot}NH_2$,HCl	–	4
M15	$\cdot NH{\cdot}CH_3$,HCl	–	0
M16	$\cdot N(CH_3)_2$,HCl	+	31
M17	$\cdot O{\cdot}[CH_2]_2{\cdot}N(C_2H_5)_2$,HCl	–	11
M18	$\cdot N^+(CH_3)_3I^-$	–	42
M19	$\cdot O{\cdot}[CH_2]_2{\cdot}N^+(CH_3)_3Br^-$	–	65

mustards with free amino acid in the medium. Calculations based on the results of chemical experiments (Fruton, Stein & Bergmann, 1946) on the reaction of M2 with phenylalanine showed that under the experimental conditions described above not more than 0·2% of the amino acid could have reacted chemically with the nitrogen mustard, even if direct contact between the two substances had been maintained throughout the period of incubation. Further evidence supporting the view that this effect is negligible is inherent in the results (Table 2) obtained with homologues in the series of aromatic nitrogen mustards having aliphatic acid side chains. Their chemical reactivities, measured in terms of carbonium ion formation, are very similar, but their biological effects measured in the staphylococcal system show great variation.

The interpretation of these results on a chemical basis must therefore take into account the interaction of the nitrogen mustards with the various cellular constituents. There is from these experiments no evidence that the nitrogen mustards actually penetrate the boundaries of the cell. It is clear that such a penetration would be followed by some reaction between the inhibitor and the cellular protein and nucleic acid, but the fact that the toxic effects of the nitrogen mustards are not marked within the short experimental period shows that the extent of the reaction cannot be sufficient to effect any important changes in the overall metabolism of the cells. However, the possibility of a specific reaction of the nitrogen mustards with a site of special importance in protein synthesis cannot be ruled out. If such a site were situated in the cell wall or cytoplasmic membrane, then the transport of amino acids into the cell could clearly be hindered; and the active transport of amino acids across cell walls and membranes must surely be envisaged as a part of the chain of events leading from amino acids to protein. To support this view, the recent work of Cowie & Walton (1956) on amino acid assimilation by *Torulopsis utilis* has shown that, in this micro-organism at least, absorbed amino acids do not exist in the free state at all but enter at once into a metabolic pool where they are associated with sites intimately concerned with protein synthesis. Certainly our results would not preclude an action of the nitrogen mustards on this first stage alone. There is a further possible explanation of our observations: the amino acids in the metabolic pool may exist in local concentrations that are sufficiently high for chemical reaction with the inhibitor molecules to proceed at rates much greater than those pertaining to the extracellular concentrations. If these rates were high enough to prevent the incorporation of a considerable proportion of the amino acid into protein, the differential activities of nitrogen mustards containing chloro-groups of similar chemical reactivities could be ascribed to different extents of penetration into the cell.

It is impossible to say whether the results obtained in this bacterial system can be correlated with results of metabolic studies on higher organisms. If the processes of protein synthesis follow similar pathways in all living cells then our results suggest that, in general, the primary inhibitory action of nitrogen mustards is to produce a block at some stage in the process of protein synthesis. On the assumption that there are certain similarities in the processes of protein synthesis in all organisms, the correlation between the results of tests on the bacterial system and of the same compounds on the Walker rat carcinoma are presented. An additional negative correlation might be mentioned at this point. The compound 1:4-dimesyloxybutane (Myleran) active against chronic myelogenous leukaemia is completely inactive in the bacterial system; nor does it react with amino acids or nucleic acids. Clinical observations, however, suggest that the mode of action is different from that of the nitrogen mustards.

It will be noticed (Table 4) that there are some apparent exceptions to the correlation. In particular it may be significant that the two quaternary compounds (M18 and M19) which showed relatively strong activity in the bacterial system are both very water-soluble. In one case at least the inactivity against the Walker carcinoma was surprising and could probably be explained by rapid excretion of the compound. Very little inhibition of amino acid uptake was found when M13 was examined (Table 4), but it has not been tested against the Walker rat carcinoma except under conditions where other toxic side effects are manifest. Thus the positive reaction that we assign to it must remain in some doubt.

Finally it should be mentioned that alterations appearing in the blood picture in the course of treatment of animals with the aromatic nitrogen mustards with aliphatic acid side chains have recently been described (Elson, 1955). The correlations with our results and with the inhibitions of the Walker rat carcinoma are again close.

SUMMARY

1. An investigation has been made of the effects produced by several aliphatic and aromatic nitrogen mustards on the incorporation of ^{14}C-labelled amino acids into protein by *Staphylococcus aureus*.

2. Different nitrogen mustards inhibited this amino acid incorporation to different extents. Where there was a pronounced inhibition, it could

not be ascribed to a direct toxic effect in any of the cases where this was studied.

3. The compounds that proved to be most effective in reducing the amount of the incorporation of amino acids into the bacterial protein were also, in general, those most effective in inhibiting the growth of the Walker rat carcinoma.

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Some Physicochemical Properties of Human Fibrinogen

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Among the numerous studies of the physicochemical properties of fibrinogen the majority of investigators have confined themselves to the more readily available bovine protein. A preparation of human fibrinogen made by salt fractionation was studied by Holmberg (1944); sedimentation velocity and diffusion measurements gave a molecular weight of 600 000 and a frictional ratio of 3; this leads to an axial ratio of 50, assuming zero solvation. Human fibrinogen prepared by the ethanol fractionation method of Cohn and co-workers had a molecular weight of 400 000 and a frictional ratio of 1·98 from sedimentation-velocity and intrinsic-viscosity determinations, and a molecular weight of 580 000 by osmotic-pressure measurements (Oncley, Scatchard & Brown, 1947). Studies on the double refraction of flow by Edsall, Foster & Scheinberg (1947) led to the conclusion that the fibrinogen molecule could be approximated in shape by a prolate ellipsoid 700 Å long with an axial ratio of 18. The material prepared by further fractionation of fraction '1' (Morrison, Edsall & Miller, 1948) always contained a small proportion of a fast component in both the ultracentrifuge and electrophoresis apparatus. Electrophoretic studies by Avery & Munro (1948) of fractions of human fibrinogen prepared by various salt-fractionation methods showed that only one of the materials prepared by precipitation with phosphate consisted of a single electrophoretic component. In a preliminary investigation of purified human fibrinogen prepared by ether precipitation (Kekwick, Mackay, Nance & Record, 1955) a molecular weight of 340 000 and a frictional ratio of 2·34 were found from sedimentation velocity and diffusion measurements (Caspary & Kekwick, 1954). This gives an axial ratio of 28 on the assumption of an unhydrated prolate ellipsoid. The preparations examined had no additional components either on electrophoresis or in the ultracentrifuge. The present paper deals with more extensive sedimentation and diffusion measurements on similarly purified human fibrinogen. The

accuracy of the measurements is discussed and the electrophoretic and ultracentrifugal boundaries are analysed. The deviations of the free diffusing boundary from the ideal are also described. Some further evidence for the suggested disaggregation of the fibrinogen molecule at low concentration (Caspary & Kekwick, 1954) is also given.

MATERIALS AND METHODS

Fibrinogen. This protein, prepared by the method of Kekwick *et al.* (1955), had clottable-nitrogen values between 97 and 98% and was essentially free from plasmin and plasminogen. The clottable nitrogen usually fell by about 1% on freeze-drying. It should be noted that a peptide is split off during the clotting process (Lorand, 1952); it accounts for about 3–3·5% of the fibrinogen nitrogen and implies that the clottability of 100% fibrinogen should be assessed as 96·5–97%. For work on freshly prepared material the product was dialysed immediately in the cold and not allowed to reach room temperature until all traces of ether had been removed; this procedure greatly increases the stability of solutions during storage at 2–4°. The total protein concentration was determined either by micro-Kjeldahl nitrogen estimation or refractometrically.

Refractive index. Refractive index was measured with a Zeiss dipping refractometer at λ 546 mμ and 25°. The specific refractive increment was arbitrarily assumed to be 0·200 ml./g.

Micro-Kjeldahl nitrogen. This was estimated in the apparatus of Markham (1942) by using the mixed bromocresol green–methyl red indicator of Ma & Zuazaga (1942).

Buffers and solutions. Citrate–saline pH 7·7: NaCl 8·8 g./l., trisodium citrate 3·7 g./l.; phosphate buffer, pH 8·0, *I* 0·2: Na_2HPO_4 0·0654 M, KH_2PO_4 0·0044 M; phosphate buffer, pH 7·0, *I* 0·1: Na_2HPO_4 0·0271 M, KH_2PO_4 0·0188 M; phosphate buffer, pH 6·2, *I* 0·1: Na_2HPO_4 0·0127 M, KH_2PO_4 0·0616 M; acetate buffer, pH 5·5, *I* 0·1: sodium acetate 0·10 M, acetic acid 0·0112 M; acetate buffer, pH 4·3, *I* 0·1: sodium acetate 0·1 M, acetic acid 0·25 M; glycine buffer, pH 8·8, *I* 0·1: glycine 0·0950 M, NaCl 0·0988 M, NaOH 0·0050 M.

Electrophoresis. Electrophoretic analyses were carried out in the Tiselius apparatus at 0°, with the edge version of the diagonal schlieren optical system and monochromatic light, λ 546 mμ. Solutions of fibrinogen, 5–10 mg./ml., were made up in buffer and dialysed against buffer at 2–4° for several days, with frequent changes. The buffers used were phosphate (pH 8, *I* 0·2, and *I* 0·1) and, on the acid side of the isoelectric point, acetate (pH 4·3, *I* 0·1). The average duration of an experiment was 40 mA hr. but several experiments were continued for periods up to 300 mA hr. to ensure maximal separation of any impurities.

Boundary-spreading experiments were made at the isoelectric point, pH 5·5 (Seegers, Nieft & Vandenbelt, 1945; Mihalyi, 1950). The fibrinogen was made up in and dialysed against acetate buffer, pH 5·5, *I* 0·1 or 0·15, and then centrifuged at 0–2° to remove any residue; the protein is not very soluble under these conditions and the maximum concentration attainable was of the order of 4 mg./ml. In order to obtain greater accuracy in tracing the electrophoretic patterns the schlieren straight edge was replaced by a slit 0·5 mm. wide. The form of the boundary was checked by a plot of log dn/dx against $(x-\bar{x})^2$, where x is the distance in the boundary and $\bar{x}$ the mean distance; non-linearity and divergencies between the two sides of the boundary curve indicate non-ideality. The apparent diffusion coefficient, D', was computed by the method of moments, and a heterogeneity constant calculated from the slope of the graph of D' against the time of electrophoresis (Alberty, 1948).

Sedimentation. All ultracentrifugal examinations were made in the Svedberg oil-turbine machine at 54 000 rev./min. (240 000 *g*) with the diagonal schlieren optical system (Philpot, 1938) and monochromatic light, λ 546 mμ. The rotor thermocouple of this machine was calibrated by observation of the melting of phenyl ether under standardized operating conditions (Cecil & Ogston, 1948). Photographs were taken on Ilford Rapid Process Panchromatic plates and measured with a travelling microscope (R. and J. Beck Ltd.), with a special adaptor for aligning the plates. The measurements were reproducible to $\pm$0·002 mm. Sedimentation coefficients were computed by the method of Cecil & Ogston (1948). The slope and standard deviation were calculated from the regression of the distance moved against the reduced time including the correction for viscosity, by the method of least squares. Correction to a liquid of the density and viscosity of water at 20° was made in the customary manner (Svedberg & Pedersen, 1940). The range of the mean temperatures of the ultracentrifuge rotor for the whole series of experiments was from 21 to 27°.

Two different assessments of the sedimentation homogeneity were made. First, the form of the boundary gradient curve was tested by plotting log dn/dx against $(x-\bar{x})^2$, where x is the position in the boundary measured from the centre of rotation, $\bar{x}$ the position of the maximum and dn/dx the gradient of refractive index obtained from the ordinates of the curve. For a Gaussian boundary this plot is linear and identical for both limbs of the curve. Secondly, the second moments, σ, of the boundary-gradient curve were computed from the measured ordinates of the sedimentation diagrams. The values of σ were corrected for the non-uniformity of the centrifugal field and for the concentration dependence of the sedimentation coefficient by the equation (Baldwin, 1953*a*):

$$\sigma^2_{corr.} = \sigma^2(1-\bar{\omega}^2St) + 4\omega K\Delta c' \int r\sigma\, dt,$$

where ω is the angular velocity, S the sedimentation coefficient, t the time measured from the estimated beginning of sedimentation, K is the constant in the linear sedimentation–concentration function, $\Delta c'$ is 0·34 times the concentration and r is the distance of the boundary from the centre of rotation. The integration is carried out graphically. A plot of σ^2 against t is linear for a homogeneous solute; heterogeneity is manifested as an upward curvature (Baldwin, 1953*a*, *b*). The standard deviation of the sedimentation-coefficient distribution was computed by fitting a quadratic equation to the σ^2–t plot by the method of least squares and then determining the slope of a plot of the apparent diffusion coefficient D' against a finally corrected time t''. To improve the accuracy of measurement in these experiments the schlieren straight edge was replaced by a 1 mm. wire.

Diffusion. The diffusion coefficients were determined by the Gouy interferometric method in a modified form of the apparatus of Gosting, Hanson, Kegeles & Morris (1949) at 25$\pm$0·01°. This apparatus has a parallel light beam through the cell with an optical lever arm of 115 cm. A standard

short-section electrophoresis cell specially selected for plane surfaces was used in all experiments. Boundaries were formed and sharpened by siphoning through a fine capillary (Kahn & Polson, 1947). To obtain higher sensitivity the blue Hg line, λ 436 mμ, isolated by an interference filter, was used; this has the additional advantage of making it possible to use fine-grain 'process' plates. Diffusion coefficients were computed from the first interference minimum by applying the Airey integral correction of Gosting & Morris (1949); this approximates to the conventional height–area diffusion coefficient, D_A. For several experiments values of the ideal maximum ordinate C_t were computed for all interference minima (Kegeles & Gosting, 1947) to give a qualitative picture of 'homogeneity'. Experiments were also analysed by the method of Akeley & Gosting (1953). Some experiments were also made by using the schlieren optical system and calculating the diffusion coefficient by both the height–area and moment methods.

Partial specific volume. The value of 0·725 determined for human fibrinogen by Armstrong, Budka, Morrison & Hasson (1947) was used in all experiments.

Viscosity. The viscosity measurements were made at 25±0·01°, with an Ostwald viscometer having a flow time for water of 130 sec. The specific viscosity/unit concentration was plotted against the protein concentration to give the intrinsic viscosity on extrapolation.

RESULTS

Electrophoretic behaviour. The electrophoresis diagram of purified human fibrinogen after migration at pH 8·0, *I* 0·2, for a period of 40 mA hr. had a single symmetrical peak on the ascending side migrating towards the anode; the descending boundary was more spread out and slightly skew (Fig. 1). On continuing an experiment of this type for 300 mA hr. the ascending peak remained as a single component with considerable asymmetry, and a very complex picture appeared on the descending side. By lowering the ionic strength to *I* 0·1 the complex form of the descending picture appeared within a much shorter period. On the acid side of the isoelectric point in acetate buffer, pH 4·3, *I* 0·1, the complexing phenomenon was observed within a very short period, 30 mA hr., with a very sharp ascending peak and the spread-out complex picture on the descending side. Migration at this pH was towards the cathode with a greatly increased mobility.

The method of reversible boundary spreading at the isoelectric point (Alberty, 1948) was used to determine the degree of heterogeneity, the value of pH 5·5 determined for the isoelectric point of bovine fibrinogen (Seegers *et al.* 1945; Mihalyi, 1950) being taken. In acetate buffer, pH 5·5, *I* 0·1, the boundary formed by human fibrinogen remained stationary, justifying examinations at this pH value. At *I* 0·1 the solubility of the protein was only 2·5 mg./ml. and on electrophoresis at 5° a heterogeneity coefficient of $0{\cdot}58 \times 10^{-6}$ was obtained (Fig. 2). A more stable solution was obtained at *I* 0·15, and in these circumstances the heterogeneity coefficient was $0{\cdot}33 \times 10^{-6}$. The experimental boundaries were slightly skew, owing either to an inherent skew distribution or more probably to boundary distortion during compensation.

Behaviour in the ultracentrifuge. The appearance of preparations of purified human fibrinogen in the ultracentrifuge was that of a sharp symmetrical peak with a slight tendency to skewness at higher concentrations (Fig. 3). In several preparations a small quantity of a heavier component (*S* 18) was present; such preparations were not used for definitive physical measurements.

The sedimentation coefficients over a range of concentrations from 2 to 10 mg./ml., in phosphate buffer, pH 8, +NaCl to give a total ionic strength of 0·35, for three preparations, are shown in Fig. 4. Extrapolation to infinite dilution by the method of least squares gives $S^0_{20,w}$ $7{\cdot}63 \times 10^{-13}$, standard error $0{\cdot}07 \times 10^{-13}$ with a correlation coefficient of 0·974. The slope of this line is equivalent to the factor *K* in the equation for the linear concentration dependence of the sedimentation coefficient ($S = S_0 + Kc$) and has the value $1{\cdot}2 \times 10^{-14}$ for this

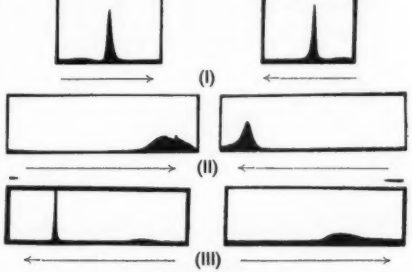

Fig. 1. Electrophoresis diagrams of human fibrinogen. (I) pH 8·0, *I* 0·2; 40 mA hr. (II) pH 8·0, *I* 0·2; 300 mA hr. (III) pH 4·3, *I* 0·1; 30 mA hr. Arrows indicate direction of migration.

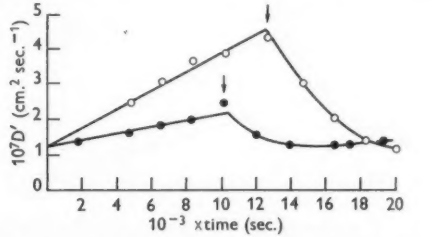

Fig. 2. Isoelectric boundary spreading of human fibrinogen. Plot of apparent diffusion coefficient against time of electrophoresis. ○, *I* 0·10; ●, *I* 0·15. Arrows indicate reversal of current.

combined experiment with a standard deviation of $0{\cdot}06 \times 10^{-14}$. Table 1 shows the values of K for the individual preparations and the combined experiment. When the 't' test was applied to these results the differences were not significant (P 0·5). No significant variations in sedimentation coefficient were found over the range of experimental temperatures (21–27°). The speed of the ultracentrifuge was held at 54 000 rev./min. and the effect of varying this was not examined.

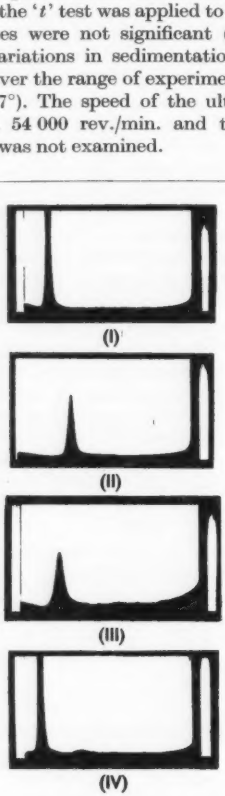

Fig. 3. Sedimentation diagrams of purified human fibrinogen. (I) 10 mg./ml., 20 min.; (II) 6 mg./ml., 30 min.; (III) 3 mg./ml., 20 min.; (IV) sample showing heavy component ($S = 18$s), 10 min. Times indicate period at 240 000 g.

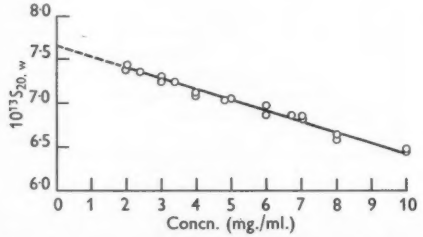

Fig. 4. Sedimentation coefficient of human fibrinogen as a function of concentration.

Bovine fibrinogen, prepared by the ether-fractionation method, in phosphate buffer, pH 8·0, with NaCl added to give a total ionic strength of 0·35, had a sedimentation coefficient of $7{\cdot}63 \times 10^{-13}$ at infinite dilution and a value for K of $0{\cdot}94 \times 10^{-14}$. It should be noted that this value of K is significantly different from that for human fibrinogen under the same conditions.

At concentrations below 1·5 mg./ml. the sedimentation coefficient of human fibrinogen preparations tended to decrease with dilution. Fig. 5 exemplifies the extremes of this type of behaviour for two preparations (F 33, F 38). Even at these low concentrations the decrease in sedimentation coefficient was well outside the limits of experimental error. The effect was eliminated or even reversed after freeze-drying or after allowing the dilute solutions to stand at 4° for 2 weeks or more (Fig. 5); it was not demonstrable with preparations having a trace of heavy component.

The effect of varying the ionic strength at pH 8·0 is shown in Table 2. The values of the sedimentation coefficient at infinite dilution are not significantly

Table 1. *Values of K in the sedimentation coefficient–concentration function for different preparations of fibrinogen*

Preparation	$10^{14}K$
F36	−1·24
F37	−1·31
F38	−1·17
F36 + F37 + F38	−1·21

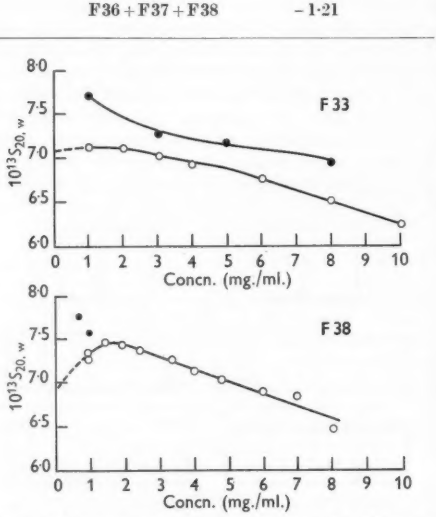

Fig. 5. Sedimentation coefficient as a function of concentration, showing anomalous behaviour at low concentration. F 33: ●, Freeze-dried; ○, fresh preparation. F 38: ●, After storage; ○, fresh preparation.

different at the lower ionic strengths, but are on the border line of significance at I 0·60. The values of K tend to decrease slightly with increasing ionic strength.

The effect of pH on the sedimentation coefficient of fibrinogen was also examined at constant ionic strength. The buffer ionic strengths were held constant at I 0·1 and 0·15 M-NaCl was added to give a total ionic strength of 0·25. The buffers used were acetate, phosphate and glycine. The variation of the sedimentation coefficient over the range pH 5·5–8·8 was barely significant; the values of K varied between 0·77 and 1·3, tending to increase with increase in pH.

It is generally considered that freeze-drying leaves the properties of proteins unchanged, but a slight increase in the sedimentation coefficient of fibrinogen occurred after freeze-drying (Fig. 5, F 33). A comparison experiment showing this effect is illustrated by Fig. 6. The values computed for the fresh material were $S_0 = 7{\cdot}65 \times 10^{-13}$ and $K = 1{\cdot}27 \times 10^{-14}$, and for the freeze-dried material $S_0 = 7{\cdot}71 \times 10^{-13}$ and $K = 1{\cdot}20 \times 10^{-14}$. The values of K were not significantly different (P 0·5) and the difference in S_0 was on the border line of significance. However, the fact that the alteration occurred with all the preparations examined tends to substantiate these findings. The sedimentation coefficient of a solution of fibrinogen (5 mg./ml.) stored at 4° and tested at intervals showed no change over a period of 30 days.

Table 2. *Effect of ionic strength on sedimentation in phosphate, I* 0·1, pH 8, *+sodium chloride*

I	$10^{13} S_0$	$10^{14} K$
0·175	7·68	−1·40
0·350	7·65	−1·26
0·600	7·76	−1·20

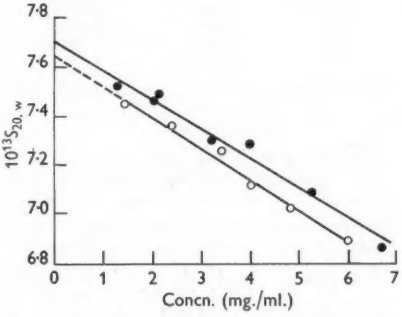

Fig. 6. Effect of freeze-drying on the sedimentation coefficient of human fibrinogen. ○, Fresh preparation; ●, freeze-dried.

On storage, fibrinogen proved to be more stable in a citrate–saline solution, pH 7·4, I 0·225, than in phosphate–saline. In citrate–saline S_0 was $7{\cdot}60 \times 10^{-13}$ and K was $1{\cdot}44 \times 10^{-14}$.

Boundary-spreading experiments in the ultracentrifuge on a fibrinogen solution in citrate–saline showed, first, that the boundary is not Gaussian, because the graph of dn/dx against $(x-\bar{x})^2$ (Fig. 7*a*) is not linear and the lines for both limbs are not coincident. Secondly, the plot of the corrected standard deviation against time (Fig. 7*b*) is not linear. By fitting a quadratic equation to derive a finally corrected time the weight-average diffusion coefficient (D_w) and the standard deviation of the sedimentation coefficient distribution (p) were computed; the values were $p = 0{\cdot}53 \times 10^{-13}$ and $D_w = 2{\cdot}4 \times 10^{-7}$ cm.^{2} sec.$^{-1}$. Analysis to derive the actual distribution was not made in view of the complications likely to arise from the disaggregation observed (Caspary & Kekwick, 1954) and the converse tendency to form a heavy component on ageing. This may also explain the relatively high

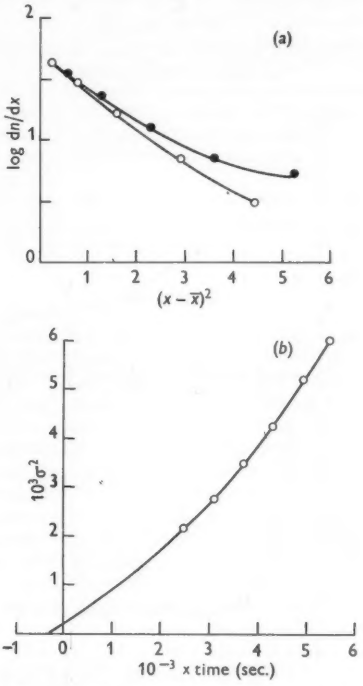

Fig. 7. Ultracentrifugal boundary spreading of human fibrinogen. (*a*) Test of the Gaussian form of both limbs of the boundary; (*b*) plot of the corrected standard deviation against time.

value of p when compared with other proteins (Baldwin, 1954). Attempts to analyse the boundaries from experiments made at low concentrations in the range 1–2 mg./ml. were unsuccessful.

Diffusion-coefficient measurements. The diffusion coefficient of human fibrinogen preparations was measured over a range of concentrations, 2–7 mg./ml. At higher concentrations accurate measurements could not be made as the magnification of the optical system was insufficient to resolve the larger numbers of fringes produced; the results for three preparations are shown in Fig. 8. The value of the diffusion coefficient D extrapolated to infinite dilution was $1{\cdot}98 \times 10^{-7}$ cm.^{2} sec.$^{-1}$, with a standard deviation of $0{\cdot}06 \times 10^{-7}$.

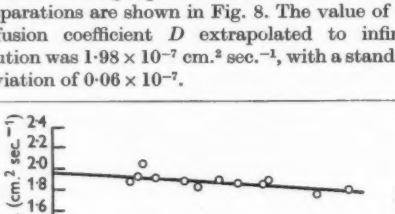

Fig. 8. Diffusion coefficient of human fibrinogen as a function of concentration.

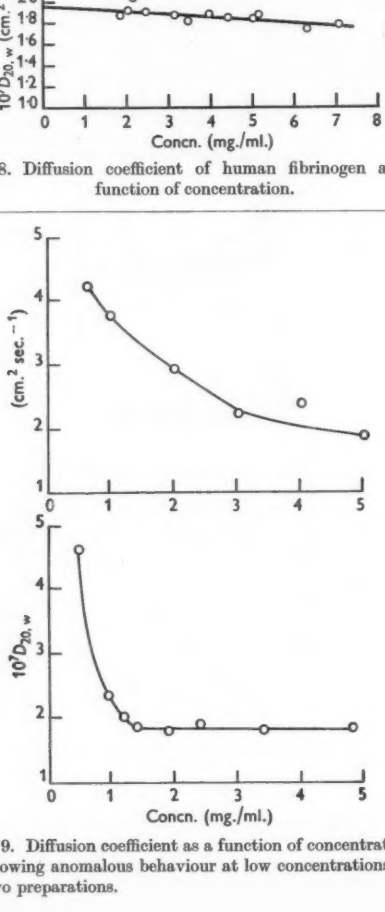

Fig. 9. Diffusion coefficient as a function of concentration, showing anomalous behaviour at low concentrations for two preparations.

At concentrations below 2 mg./ml. there was a sharp rise in the diffusion coefficient, deviating from the linear behaviour with respect to concentration; Fig. 9 shows the extremes of this effect for two preparations. The increase does not occur with freeze-dried preparations and disappears when fresh solutions are stored for several weeks.

The reproducibility of the Gouy diffusion experiments was of the order of 2–3 %. The value of D calculated from the first minimum is a close approximation to the height–area diffusion coefficient.

The boundary characteristics were checked by two methods. Values of the computed maximum ordinate, C_t, were calculated for interference minima covering the complete pattern. The results at two time intervals are given in Table 3. The values of C_t are more nearly constant at the longer

Table 3. *Computation of the theoretical maximum ordinate, C_t, after two different time intervals of diffusion*

Preparation F38, 5·5 mg./ml. j_m (total no. of interference fringes) = 57·24.

	C_t	
j (fringe no.)	9000 sec.	21600 sec.
0	1·6055	1·0370
2	1·6036	1·0360
4	1·5993	1·0333
6	1·5952	1·0330
8	1·5941	1·0317
10	1·5910	1·0307
12	1·5884	1·0299
14	1·5874	1·0305
16	1·5850	1·0292
18	1·5831	1·0276
20	1·5796	1·0285
22	1·5775	1·0260
24	1·5759	1·0266
26	1·5748	1·0254
28	1·5695	1·0253
30	1·5702	1·0252
34	1·5676	1·0236
38	1·5607	1·0222
42	1·5514	1·0231
46	1·5409	1·0329
50	1·5106	1·0451
Max. variation ...	0·095	0·015

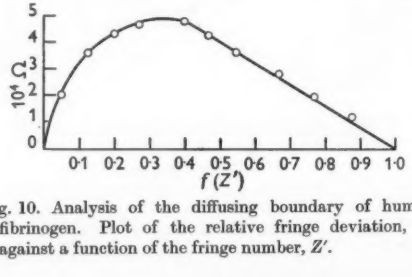

Fig. 10. Analysis of the diffusing boundary of human fibrinogen. Plot of the relative fringe deviation, Ω, against a function of the fringe number, Z'.

interval, an effect probably due to the smoothing-out of some slight boundary disturbances. The final analysis of the diffusing boundary was made by the application of the method of Akeley & Gosting (1953); Fig. 10 shows the plot of the relative fringe deviation against a function of the fringe number; the curvature of this plot may be interpreted as indicating heterogeneity.

Measurements of the diffusion coefficient with the schlieren optical system gave $D_A = 2{\cdot}4 \times 10^{-7}$ and $D_m = 2{\cdot}7 \times 10^{-7}$ cm.^{2} sec.$^{-1}$. The difference between the two average values suggests some heterogeneity; the absolute values of the coefficients tend to be high, because of greater boundary distortion caused by the method of boundary formation and compensation in this apparatus, in comparison with the interferometric apparatus.

In view of the limited accuracy of the methods and the low values of the constant in the linear concentration function for diffusion, no investigations of the concentration dependence of the diffusion of fibrinogen under various conditions were made.

Molecular weight. The molecular weight, calculated from the sedimentation and diffusion coefficients (Gouy values) extrapolated to infinite dilution, by using the Svedberg equation and a partial specific volume of 0·725, was 341 000 with a standard deviation of 10 000. The frictional ratio was 2·34. The values of the molecular weight calculated from the coefficients at corresponding concentrations over the range studied show a sharp drop in molecular weight below a concentration of 1·5 mg./ml. (Fig. 11).

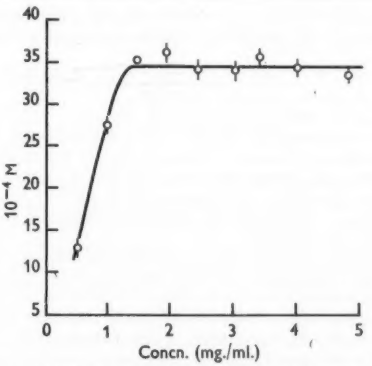

Fig. 11. Molecular weight of human fibrinogen as a function of concentration.

Table 4. *Analysis of fractions obtained by preparative ultracentrifuging of a solution containing the heavy component*

Material	Percentage of fraction	
	$S = 7$s	$S = 18$s
Initial	90	10
Supernatant	93	7
Sediment	62	38

Some observations on the $S = 18$s component. A heavy component (S 18s) forms when solutions of fibrinogen are held at 2–4° for about 10 days, without altering the clottability of the preparation. It was concluded that this component might be one of the intermediate polymers in the fibrinogen–fibrin transformation. After addition of sufficient urea to bring the concentration to 3 M (Ferry, Katz & Tinoco, 1954) or NaBr to M (Sturtevant, Laskowski, Donnelly & Scheraga, 1955), this component could no longer be detected in preparations originally containing it. Differential ultracentrifuging at 105 000*g* (Spinco Model L) for 150 min. of a preparation containing the heavy component followed by ultracentrifugal analysis of the separated materials gave the results shown in Table 4. Calculations from the proportions of the $S = 7$s and $S = 18$s in the starting material and in the fractions indicated that all the protein originally present as heavy component should have been recovered in the precipitate; this, however, contained less heavy component than was expected; when the analysis was repeated after the solution had been kept for 3 days an even smaller proportion of the heavy component was detected. This suggests that the system is in a slowly reversible equilibrium and that the quantities of the components will tend to reach a state of constant proportion.

Viscosity. The value for the intrinsic viscosity determined at 25° over the concentration range 1–9 mg./ml. was 0·245.

DISCUSSION

It is clear from the experimental results that the molecular kinetic behaviour of the most highly purified preparations of human fibrinogen is extremely complex. Fresh preparations when subjected to electrophoresis for a period adequate for the resolution of the major constituents of human plasma display a single peak only, but the descending boundary eventually becomes very complex in contour when the electrophoresis is continued for six times this period. Similarly, such preparations in the ultracentrifuge give rise to a single peak only; however, at fibrinogen concentrations below 2 mg./ml. the sedimentation coefficient drops sharply away from the linear relationship with concentration observed at higher concentrations. Correspondingly, the diffusion coefficient rises sharply and by combining the two sets of results the molecular-weight values obtained suggest that a disaggregation may be occurring involving a rapid readjustment of equilibrium.

When such fibrinogen solutions are stored for two to three weeks, or are freeze-dried and then reconstituted, this characteristic disappears and there is a tendency for a polymer of sedimentation coefficient approximately 18s to be formed. That this is also probably in equilibrium with the major component ($S = 7$s) is shown by the analysis of the supernatant and sediment from the differential preparative ultracentrifuge experiment, and also from the effect of adding urea or NaBr to solutions exhibiting heavy component. In this instance, however, the rate at which equilibrium is attained appears to be much slower than in the possible disaggregation discussed above.

The heterogeneity shown by electrophoretic and ultracentrifugal boundary-spreading measurements could be explained either by the intrinsic heterogeneity of the protein molecules (Colvin, Smith & Cook, 1954; Ogston, 1955) or as a manifestation of the disaggregation and aggregation phenomena. It has been shown by immunological methods (Caspary, 1956) that the purified fibrinogen preparations are contaminated with trace amounts of other plasma proteins, but it is unlikely that these could account for the heterogeneity demonstrated.

If the values of the sedimentation and diffusion coefficients observed at low concentrations in fresh fibrinogen preparations are accepted as indicating a disaggregation of the molecule, weight 341000, the number of subunits formed from each molecule must be at least three and could possibly be four.

SUMMARY

1. On prolonged electrophoretic migration purified human fibrinogen showed complex behaviour. Slight heterogeneity was demonstrated by isoelectric boundary spreading.

2. Ultracentrifugal examination of human fibrinogen was made under various conditions of protein concentration, pH and ionic strength. The fall of the sedimentation coefficient at low concentrations suggests a disaggregation in rapid equilibrium. Some heterogeneity was demonstrated by boundary analysis.

3. The diffusion coefficient of fibrinogen was determined. Its increase at low concentrations suggests disaggregation. The diffusion boundaries deviate from the ideal form.

4. The molecular weight of the undissociated molecule was found to be 341000 ± 10000.

5. Bovine fibrinogen showed a difference in variation of the sedimentation coefficient with concentration from that of human fibrinogen, although the sedimentation coefficients at infinite dilution were the same.

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Preparation and Characterization of the Isomaltodextrins

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In the course of studies on the amylopectin-debranching enzyme, R-enzyme (Hobson, Whelan & Peat, 1951; Peat, Whelan, Hobson & Thomas, 1954), it became necessary to prepare individual members of the series of glucose oligosaccharides containing the amylopectin-type branch linkage as the sole polymeric bond, i.e. the isomaltodextrins. The opportunity to do this was provided by the acquisition of a *Leuconostoc mesenteroides* dextran containing a very high proportion (96%, determined by periodate oxidation) of α-1:6-linkages. Acid-catalysed fragmentation of this polyglucose to an appropriate degree should provide a mixture of small isomaltodextrins, capable of being fractionated by charcoal chromatography. This expectation has been realized and the individual members within the series isomaltose–isomaltohexaose have been isolated and characterized. The last three members, isomalto-tetraose, -pentaose and -hexaose, have not previously been described, except in terms of R_F value.

The oligosaccharides described have been used in studies of the inhibition of precipitation of dextran by human antidextran (Kabat, 1956).

RESULTS

Hydrolysis of the dextran

The dextran was hydrolysed in dilute sulphuric acid until the apparent conversion into glucose was 52%, a value chosen on the basis of the Kuhn (1930) formula as appropriate for the maximum yield of tri- to penta-saccharides. Fractionation and re-fractionation on charcoal–Celite led to the separation of glucose, as the only monosaccharide component, and five amorphous sugars of decreasing R_F value, each of which migrated as a single zone during paper-chromatographic fractionation. The oligosaccharides were also of high purity in respect of freedom from non-carbohydrate material (Table 1).

Properties of the isomaltodextrins

Molecular weight. The molecular weights of the dextrins were determined by comparison of their copper-reducing powers, the assumptions being made that each molecule contains only one free-reducing group and that the molecular weight of the lowest member, isomaltose, is 342. The results are in excellent agreement with expectation (Table 1).

Optical rotation. The molecular rotations of the dextrins showed the expected linear relationship with degree of polymerization (Fig. 1, Whelan, Bailey & Roberts, 1953; Lindberg & McPherson, 1954; French, 1955). Glucose does not conform to this relationship, an observation already made with the maltodextrins (Whelan *et al.* 1953), the laminaridextrins (Lawley, 1955) and the gentiodextrins (Haq & Whelan, 1956), indicating that the disaccharide must be regarded as the lowest member of any such polymeric series.

Jones, Jeanes, Stringer & Tsuchiya (1956) have described the preparation and properties of the methyl α-glycosidic derivatives of isomaltose, isomalto-triose, -tetraose and -pentaose. It is to be expected that the molecular rotational increment/glucose residue in this series should be the same as in the series of free sugars. The calculated value for the glycosides is +318°; for our series the value is

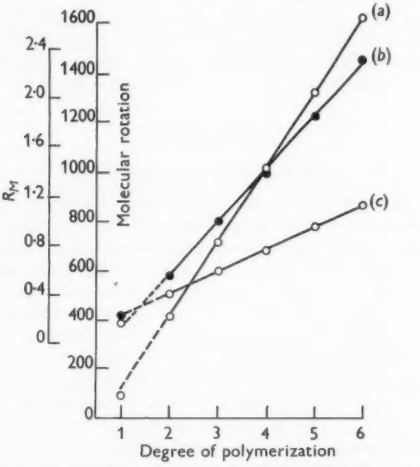

Fig. 1. Relations between degree of polymerization of the isomaltodextrins and (*a*) molecular rotation, (*b*) and (*c*) R_M values (see text) calculated from R_F values determined in butanol–pyridine–water (6:4:3, by vol.) and propanol–ethyl acetate–water (6:1:3, by vol.) respectively, by descending chromatography on Whatman no. 54 paper.

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Table 1. *Properties of the isomaltodextrins*

The yields are those from 54 g. of dextran; 21·1 g. of glucose was also obtained. Charcoal–Celite column (Whelan *et al.* 1953) 95 cm. × 5 cm. The elution volume is the volume of eluate in which the sugar appeared during refractionation by gradient elution (see Experimental section).

Substance	Elution vol. (l.)	Yield (g.)	Carbohydrate content (%)	$[\alpha]_D$ in water	Mol.wt. Found	Mol.wt. Calc.	Ester derivative m.p.	Ester derivative $[\alpha]_D$ in $CHCl_3$
Isomaltose	—	9·3	—	+122°	—	342	143·5–144·5°*	+97°
Isomaltotriose	7·0–9·2	4·0	97·1	+142†	500	504	226–227‡	+131
Isomaltotetraose	10·8–12·6	2·2	98·1	+153	672	666	176–179‡	+166
Isomaltopentaose	14·2–16·2	2·4	98·6	+160	826	828	—	—
Isomaltohexaose	17·8–18·8	0·58	95·2	+163	987	990	—	—

* Acetyl derivative; values for authentic specimens are given by Bacon & Bacon (1954).
† Jeanes *et al.* (1953) quote +145·0° for this substance; Wolfrom & Thompson (1956) quote +128°.
‡ Benzoyl derivative.

+300°. The average increments in the series of methyl αβ-glycosides prepared by methanolysis of a similar dextran is +367° (Scott & Senti, 1955). Possibly the large difference from the foregoing values is due to an inconstant ratio of the α- and β-forms in the series of methyl glycosides.

R_F values. The R_M values, log $(1/R_F - 1)$, of the isomaltodextrins in two solvent systems are plotted against degree of polymerization in Fig. 1. The expected linear relationships were again observed (cf. Whelan *et al.* 1953; French & Wild, 1953).

Esters. Isomaltose can be characterized as the crystalline octa-acetate but isomaltotriose acetate could not be crystallized, either by Jeanes, Wilham, Jones, Tsuchiya & Rist (1953) or by the present authors. Recourse was had to the preparation of benzoyl derivatives, and crystalline specimens of β-isomaltotriose undecabenzoate and β-isomaltotetraose tetradecabenzoate were obtained (Table 1).

DISCUSSION

Structure of the isomaltodextrins

The properties of the disaccharide and its octa-*O*-acetyl derivative were identical with those reported for isomaltose [*O*-α-D-glucopyranosyl-(1 → 6)-D-glucose]. The properties of the higher dextrins, namely molecular weights, molecular rotations and R_M values, are entirely consistent with their being members of the polyglucose series based on the repeating α-1:6-linkage and, in addition, the trisaccharide had the same specific optical rotation as that already reported for isomaltotriose (see Table 1). The structures of the tri- to hexa-saccharides are therefore established.

On the evidence that only α-1:6-linked glucose polymers were obtained from the dextran hydrolysate, it might be concluded that the original polysaccharide molecule contained only these linkages. As already mentioned, periodate oxidation indicated that 96 % of the linkages were of the 1:6-type. Recent methylation studies by American workers have, however, shown that a similar dextran, produced by the NRRL B-512 strain of *Leuconostoc mesenteroides* and having 95 % of 1:6-links (by periodate oxidation: Jeanes & Wilham, 1950; Rankin & Jeanes, 1954; Dimler, Wolff, Sloan & Rist, 1955), also contained about 5 % of 1:3-bonds (Van Cleve, Schaefer & Rist, 1956). These additional linkages apparently constitute points of branching. If such linkages were also present in our dextran it is unlikely that they would cause contaminants, in the way of 1:3-linked sugars, to be present in the purified oligosaccharides, even to the extent of only 5 %. There are three reasons for this statement: First, the 1:6-link is more resistant to acid hydrolysis than the 1:3-link. The breakdown products will therefore contain fewer than 5 % of 1:3-links. Secondly, the paper- and charcoal-chromatographic separations of isomaltose from nigerose (α-1:3-link) or laminaribiose (β-1:3-link) are very marked, and such contaminants would therefore be easy to recognize. Thirdly, in fractionation of the dextran hydrolysate the fractions representing the beginning and end of the elution of a sugar were deliberately rejected to ensure the purity of the major product. Our results do not therefore allow us to conclude anything about the structure of the polysaccharide, other than that the great majority of the polymeric linkages must be of the α-1:6-type.

Reducing powers of the isomaltodextrins

Whelan *et al.* (1953) showed that the molar-reducing power towards Somogyi (1945*a*) copper reagent in the series maltose–maltoheptaose was constant, but in order to develop the full reducing power of each maltodextrin it was necessary to heat it with the copper reagent for a period which increased with the molecular size of the dextrin. Maltose required 20 min. heating and maltoheptaose 45 min. By contrast, the isomaltodextrins

all required the same time to develop full reducing power, namely 30 min. A similar observation has been made with the 1:6-linked polymers of β-D-glucose (Haq & Whelan, 1956).

This information is relevant to the attempts that have been made to determine the molecular weights of polysaccharides by measurements of copper-reducing power. Such methods are notoriously unreliable for the large starch-type polymers containing the maltose linkage, and the results obtained by Whelan *et al.* (1953) offer an explanation of this unreliability. Isbell, Snyder, Holt & Dryden (1953) obtained good agreement between molecular weights of dextrans as measured by copper-reducing power, calibrated against isomaltose, and molecular weights measured by other methods. The present results are in agreement with these observations.

EXPERIMENTAL

Preparation of the dextran

The dextran was a purified, partly degraded specimen with an approximate weight-average molecular weight of 180 000, elaborated by the NRRL B-512 strain of *Leuconostoc mesenteroides*. This type of dextran and the organism have been described by Jeanes, Wilham & Miers (1948).

Hydrolysis of the dextran and fraction of the products

The dextran (54 g.) was dissolved in hot water (1 l.), and 6N-H_2SO_4 (277 ml.) and water were added to give 5 l. of solution, 0·33N with respect to acid. This was heated under reflux on a boiling-water bath and samples were removed at intervals for determination of copper-reducing power, as glucose (Somogyi, 1945*a*). After 10 hr., when the reducing power corresponded to 52% conversion into glucose, the solution was cooled and neutralized to pH 6–7 with NaOH solution. The hydrolysate was absorbed on a charcoal–Celite column (95 cm. × 7·3 cm.), prepared as by Whelan *et al.* (1953), and fractions (500 ml. each) were collected during elution with water and then with stepwise-increasing concentrations of ethanol in water (5, 8, 15, 20 and 50%, v/v). The optical rotations of the fractions were measured in a 4 dm. tube. Glucose (21·1 g.) appeared in the fractions corresponding to 7·5–27·5 l. of water. Ethanol (5%) was then added. Fractions corresponding to 28–36·5 l. (1·2 g.) contained a mixture of glucose and isomaltose, while fractions corresponding to 37–48 l. (10·3 g.) contained isomaltose with a trace of glucose. The higher fractions were not obtained in a sufficiently pure state, with respect to freedom from other sugars, and they were combined and refractionated on a charcoal–Celite column (95 cm. × 5 cm.) by gradient elution, 30% aqueous ethanol being fed through a constant-head device into a reservoir of water (15 l.), which in turn was connected to the column. Fractions (200 ml. each) were collected and their optical rotations determined as before. Fractions representing the peaks of optical activity were combined, evaporated to dryness, and freed from inorganic matter as by Whelan *et al.* (1953). The isomaltose was refractionated on charcoal–Celite to remove the traces of glucose, and the weights of chromatographically pure fractions are shown in Table 1.

Properties of the isomaltodextrins

Isomaltodextrin solutions used for analytical determinations were first treated with Somogyi's (1945*b*) deproteinizing reagents, as by Whelan *et al.* (1953), to remove substances interfering with reducing-power determinations.

Carbohydrate content and specific optical rotation. The carbohydrate contents of the fractions were determined by acidic hydrolysis to glucose under the conditions defined by Pirt & Whelan (1951), except that the time of hydrolysis was increased to 10 hr., experiments having shown that isomaltotriose was not completely hydrolysed after the usual 2 hr. heating period and that the reducing power of the hydrolysate gradually increased to a maximum at 10 hr. heating. The appropriate correction factor was applied for loss of glucose due to acidic degradation and reversion (Pirt & Whelan, 1951). The weight of isomaltodextrin present was calculated on the basis of its molecular size as judged by the R_M value (Fig. 1). The carbohydrate contents of the specimens are listed in Table 1.

Specific optical rotations were calculated from measurements (4 dm. tube) on isomaltodextrin solutions of which the concentrations had been determined by acidic hydrolysis.

Molecular weights. When isomaltopentaose was heated with Somogyi (1945*a*) copper reagent for times varying from 20 to 60 min. the reducing power was found to be constant between 30 and 60 min., and at 20 min. was less than 0·5% below the maximum value. A time of 30 min. was chosen and the reducing powers of isomaltodextrin solutions of known concentration were measured. The molecular weights were calculated on the assumption that the molecular weight of the disaccharide was 342 and that each dextrin contained only one free reducing group (Table 1).

Crystalline ester derivatives

Isomaltose (204 mg.) was acetylated with acetic anhydride–sodium acetate to yield the crystalline β-octa-acetate (yield, 63%; Table 1). The m.p. was not depressed on admixture of the product with an authentic specimen.

Isomaltotriose (238 mg.) was dissolved in warm pyridine (5 ml.), and benzoyl chloride (1·5 ml.) was added slowly to the cooled solution, the temperature being kept below 40°, after which the stoppered vessel was shaken for 24 hr. at 0–5° and finally refluxed at 120–130° for 1 hr. The solution was poured into iced water (150 ml.), and the mixture shaken at 0° for 24 hr. and extracted with chloroform (3 × 50 ml.); the combined extracts were washed with water, dilute $NaHCO_3$, and water, then dried (Na_2SO_4). The solution was evaporated to a syrup which was decolorized in ethanolic solution with charcoal and left to crystallize. Recrystallization from ethanol was unsuccessful but several recrystallizations of the coarse white needles were made from ethanol–acetone (1:1, v/v). The yield was 308 mg. (40%) of *β-isomaltotriose undecabenzoate* (Table 1) (Found: C, 68·75; H, 4·6. $C_{95}H_{76}O_{27}$ requires C, 69·2; H, 4·6%).

Isomaltotetraose (200 mg.) was treated similarly and an amorphous product was obtained from ethanol–acetone. This was crystallized from ethanol–benzene (1:1, v/v), to which water had been added until turbidity appeared, and cleared by the addition of ethanol. On slow evaporation at room temperature fine colourless needles were deposited, together with some amorphous material. The same solvent

system was used for recrystallization. The yield was 432 mg. (69%) and the properties of the *β-isomaltotetraose tetradecabenzoate* are listed in Table 1 (Found: C, 68·6; H, 4·5. $C_{122}H_{98}O_{35}$ requires C, 69·0; H, 4·65%).

Attempts to prepare a crystalline octabenzoate of isomaltose were unsuccessful.

SUMMARY

1. A dextran synthesized by *Leuconostoc mesenteroides* (strain NRRL B-512) has been partly hydrolysed with acid and the products were fractionated on charcoal–Celite.

2. Glucose was the only monosaccharide product and the di- to hexa-saccharide fractions each contained a single component.

3. The oligosaccharides have been characterized as the lower members of the isomaltodextrin series and new crystalline derivatives of the tri- and tetra-saccharides have been prepared.

4. Some observations on the copper-reducing powers of glucose oligosaccharides are recorded.

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The Determination of 10–100 μmg. Quantities of Hexosamine

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The most sensitive methods for the estimation of hexosamine described so far are those of Elson & Morgan (1933) as modified by Blix (1948), and of Dische & Borenfreund (1950). During the course of investigations in this laboratory it became necessary to determine as little as 10 μmg. of hexosamine in 0·2–0·5 μl. samples of the endolymph of the inner ear. Ultramicro modifications of the above methods allow at best quantities of 40 μmg. of hexosamine to be determined, and were therefore not sufficiently sensitive.

The present paper describes a more sensitive and more specific method for the determination of hexosamine, which allows 10–100 μmg. to be determined. The bound hexosamine is liberated by acid hydrolysis, and separated from sugars and amino acids by treatment with an ion-exchange resin from which it is eluted with hydrochloric acid. The hexosamine is then deaminated by treatment with sodium nitrite in acetic acid to give hydroxymethyldihydroxytetrahydrofurfuraldehyde (2:5-anhydrosugars) after a Walden inversion at $C_{(2)}$ of the hexosamine. Degradation of these stable hydrofuran derivatives by hydrochloric acid yields laevulic and formic acids, through the intermediates hydroxymethylfurfuraldehyde and hydroxylaevulic aldehyde (Fig. 1) (Newth, 1951). Reaction between 2:5-anhydrosugars and various

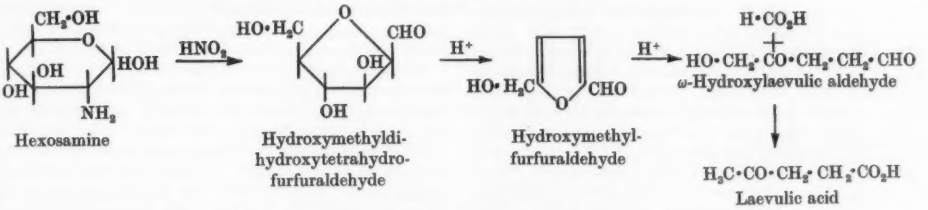

Fig. 1. Deamination and acidic degradation of hexosamine with conc. HCl.

chromogenic reagents used for estimating aldehydes were tried, and a new colour reaction produced by pyrrole in concentrated hydrochloric acid was found to be the most sensitive.

The absorption of the yellow colour was measured with ultramicrocapillary absorption cells. A modification of the method of Elson & Morgan suitable for determining amounts as low as 30 μmg. is also described.

EXPERIMENTAL

Pipettes and burettes. The ultramicro-pipettes and -burettes were those described in a previous paper (Exley, 1956). All pipettes except those used for delivery of acid were treated with DC 1107 silicone fluid (Midland Silicone Co., London).

Reaction tubes. Except with hydrolysis in small sealed tubes the reactions were carried out in small Pyrex-glass reaction tubes 5 cm. long, internal diam. 2·5 mm. (calibrated for 200 μl. vol.) fitted with ground-glass stoppers.

Stirrers. Two types of stirring were used. Solutions were stirred with either a pin stirrer consisting of a 5 cm. length of Pt wire attached to the protruding prong of an electric razor, inserted into the solution to be stirred (Exley, 1956), or a solenoidal vibrator consisting of a vibrating arm which struck the reaction tubes 50 times/sec.

Pipette holder. This was used for the delivery of samples and reagents into the reaction tubes and was similar to that described by Holter (1943).

Reagents. Amberlite IRC-50 resin was adjusted to pH 7 with McIlvaine buffer (1921). The hydrogen form of the resin was first treated with 4% (w/v) NaOH (10 ml./5 g.), shaken for 10 min. and filtered off by a Büchner flask and funnel. The resin was then washed with 30 ml. of water, 20 ml. of buffer was added and the mixture was shaken for 10 min. and filtered. The resin was again washed with 20 ml. of water. The hexosamine standard was made from pure recrystallized glucosamine hydrochloride (British Drug Houses Ltd.); it was stored at $-10°$. Aldehyde-free acetic acid was prepared by distilling A.R. acetic acid over 2:4-dinitrophenylhydrazine and taking the middle fractions. A 17·5% (w/v) solution of A.R. $NaNO_2$ was made up fresh for each series of determinations. Ammonium sulphamate (C.P.) was recrystallized and a 25% (w/v) solution prepared. A 0·25% solution of A.R. $SnCl_2,10H_2O$ in conc. (36%, w/w) HCl was made up fresh for each series of determinations. The pyrrole reagent consisted of 1% (v/v) fresh-distilled pyrrole in redistilled A.R. methanol. This was made up fresh for each series of determinations and kept in a brown bottle at 0° when not in use. A 3% (v/v) solution of redistilled acetylacetone in 1·5N-Na_2CO_3 (A.R.) was made fresh for each series. The Ehrlich reagent consisted of 2·5 g. of thrice-recrystallized *p*-dimethylaminobenzaldehyde dissolved in 30 ml. of redistilled ethanol and 30 ml. of 36% (w/w) HCl.

Liberation of hexosamine by hydrolysis. The volume of the sample was measured by a technique described previously (Exley, 1956).

For hydrolysis 20 μl. of HCl of strength suitable for the type of hydrolysis required was pipetted either into a reaction tube or into a tube which was later to be sealed. The ultramicropipette was held in the special holder and manipulated so that it did not touch the sides; the tip was placed immediately above the bottom of the tubes. The sample (0·2–0·5 μl.) was introduced by another pipette and without touching the sides was blown by mouth into the HCl. The sample pipette was washed out by alternately sucking and blowing out the liquid in the tube. The pipette was removed by lowering the pipette-holder stand. The contents of the tube were now stirred by a pin stirrer and then centrifuged at 3000 *g* for 5 min. The stoppers were fitted tightly, or the tubes were sealed and placed in a boiling-water bath for the hydrolysis; the sealed tubes were totally immersed in the boiling bath.

Acid mucopolysaccharides such as chondroitin sulphuric acid and hyaluronic acid were hydrolysed with either 4N-HCl in sealed tubes for 4 hr. as described by Johnston, Ogston & Stanier (1951) or with 4N-HCl in stoppered tubes for 12 hr. Blood-group substances were hydrolysed with 2N-HCl for 2 hr. and serum with the same strength for 12 hr. (Boas, 1953). Optimum conditions for maximal yields of hexosamine were determined for other substances by hydrolysing with concentrations of HCl up to 6N in a boiling-water bath and varying the times of hydrolysis between 2 and 15 hr. The conditions finally used are given in Tables 2, 4 and 5.

The destruction of hexosamine that occurred during the hydrolysis of acid mucopolysaccharides was estimated as follows. A sample of the material was hydrolysed under conditions found to give maximum yield and the amount of hexosamine estimated (sample value). Next, another sample to which was added an approximately equal amount of glucosamine was hydrolysed under the same conditions and the total amount of hexosamine estimated (total value). The difference between the total and sample values should equal the amount of hexosamine added if no destruction occurred. The amount by which the difference varied from the added hexosamine must be due to the destruction of added hexosamine during hydrolysis. This was calculated as a percentage of added hexosamine.

Resin separation. After hydrolysis the tubes were removed from the water bath, dried externally with tissue paper and centrifuged for 15 min. at 3000 *g*. The tubes were opened or the stoppers were removed and their contents frozen in a beaker of solid CO_2. As soon as they were frozen they were placed in a small beaker containing solid dry KOH pellets, which was placed in a desiccator also containing KOH. The desiccator was evacuated to 0·5 mm. mercury pressure with an oil pump at such a rate that no rapid boiling took place. The tubes were then left in a desiccator for several hours until completely dry. The insides of the tubes were next washed down with three lots of 10 μl. of water and the contents were stirred by a pin stirrer. The stoppers were replaced and the tubes centrifuged for 10 min. at 3000 *g*. The contents of the sealed tubes were now transferred to the reaction tubes. It was essential at this stage to ensure that the pH was not lower than 4 by testing a small sample (0·02–0·05 μl.) removed with a very fine glass thread and spotted on universal indicator paper. A few grains (about 10 mg.) of buffered IRC 50 resin were placed halfway down the tubes by a miniature glass spatula, and the tubes again centrifuged. The stoppers were replaced, the tubes vigorously vibrated by the solenoidal vibrator for 10 min., and centrifuged for 3 min. at 3000 *g*, again vibrated for 10 min. and finally centrifuged. The supernatant was removed by a finely drawn-out pipette whose bore at the tip was finer than the smaller grains of resin. The supernatant was discarded and the resin washed with 10 μl. of water, which was also discarded. To extract the hexosamine 20 μl. of N-HCl was added to the resin and the tubes were centrifuged for 3 min. They were then vibrated against the solenoidal vibrator for 10 min., centrifuged for 3 min. and vibrated again for 10 min.

The HCl extract was removed by a centrally held pipette which had not been treated with silicone. The extract was placed in a clean reaction tube, the resin washed with 15 μl. of water and the washing added to the first extract. The tube was then centrifuged for 5 min. at 3000 *g*.

Deamination. A 10% (w/v) solution (10 μl.) of Na_2CO_3 was added to the tube. After 5 min. the contents were stirred, centrifuged and 5 μl. of acetic acid and 5 μl. of freshly prepared $NaNO_2$ solution were added. The contents were now stirred with a pin stirrer, centrifuged for 2 min. and left for 10 min. until deamination was complete. After deamination 10 μl. of ammonium sulphamate solution was added in two portions of 5 μl., and after 5 min. the whole stirred again by the pin stirrer. The tubes were stirred for 1 min. every 5 min. for a period of 20 min.

Colour development and colorimetry. The $SnCl_2$–conc. HCl reagent (135 μl.) and 0·5 μl. of 1% pyrrole solution were added to the tubes, and the contents were stirred by the pin stirrer. The stoppers were replaced and the tubes placed in a water bath at 60° ± 1° for 5 min. (accurately timed), and then immediately cooled in ice until absorptiometric measurement. The colour is stable under these cooled conditions for 2 hr.

The contents were then placed either in cells of 1 cm. light path (Bessey & Lowry, 1946; Hughes, 1951) or in 2 mm. capillary cells of 160 μl. volume (Exley, 1956), and absorption was measured in a Beckmann DU spectrophotometer at 433 mμ. Unknowns were measured against a blank and standards which had been put through the full procedure. Solutions containing less than 100 μmg. were measured in the capillary cells; samples containing more than 100 μmg. were measured in the Bessey & Lowry (1946) cells. The capillary cells were filled as described by Exley (1956) and aligned as described by Kirk (1950).

Ultramicro modification of the method of Elson & Morgan. The sensitivity of the method of Elson & Morgan (1933) as modified by Blix (1948) was improved by increasing the concentration of the Na_2CO_3 in the acetylacetone reagent to 1·5 N, and the amount of *p*-dimethylaminobenzaldehyde to 2·5 g./60 ml. of ethanol–HCl. It was found that maximum colours were produced when the final concentration of ethanol was 50%. The modified method was adapted to the ultramicro scale.

After separation by resin as already described and addition of 10 μl. of 10% (w/v) Na_2CO_3, 45 μl. of a solution of 3% acetylacetone in 1·5 N-Na_2CO_3 was added. The contents were stirred by a pin stirrer and centrifuged for 5 min. at 3000 *g*. The stoppers were returned to make a tight fit and the tubes heated for 30 min. in a boiling-water bath. After heating, the tubes were cooled in ice, and 90 μl. of ethanol was added, the contents were stirred and then 20 μl. of *p*-dimethylaminobenzaldehyde reagent was added slowly. The tubes were next left for 5 min., restirred and then left for 30 min. before the absorption was measured at 530 mμ. Measurement was made either in capillary cells or Bessey & Lowry cells.

Micro methods. The ultramicro pyrrole procedure and the ultramicro Elson & Morgan (1933) method were used on the micro scale by increasing the volume of the reagents 30 times, to a final volume of 6 ml. Absorptiometric measurement was made in 1 cm. cells. These procedures are referred to as the micro pyrrole method and the Elson & Morgan micro method, respectively.

RESULTS AND DISCUSSION

Liberation of hexosamine by hydrolysis

Both the method of Elson & Morgan (1933) and the present method depend on the presence of free amino and aldehydic groups for chromogen formation. In biological material, therefore, the combined hexosamine must be freed by acid hydrolysis. The criteria of choice of optimum time and strength of acid for complete hydrolysis is that the quantity of hexosamine and the reducing power does not change after reaching a maximum. Hydrolysis of acid mucopolysaccharides under these conditions is accompanied by some destruction of the freed hexosamine. Table 1 shows some results obtained for the destruction of hexosamine in the presence of acid mucopolysaccharides. A larger number of results show that the destruction was 12 ± 2% when 4 N-acid was used for 4 hr. in sealed tubes, and 5 ± 1% with 4 N-acid for 12 hr. in stoppered tubes. These values, based on the destruction of added free glucosamine, are not necessarily accurate for combined glucosamine, since its lability may vary with its state of combination. The difference between the losses observed and the lower values obtained by Johnston *et al.* (1951) under the same conditions may be due to some factor concerned

Table 1. *Destruction of hexosamine in the presence of acid mucopolysaccharides*

Hexosamine in the biological materials below was estimated by the Elson & Morgan (1933) micro method (see Experimental section). The sources of the materials are given in Table 6. Hexosamine was calculated as $C_6H_{13}O_5N$. With the polysaccharides the amount given for hexosamine content is that found by colorimetric estimation and therefore is lower than the absolute value by the amount lost owing to acid destruction.

Material	Condition of hydrolysis: HCl concn. (N)	Condition of hydrolysis: Time (hr.)	Hexosamine content (μg.)	Amount of hexosamine added (μg.)	Total amount of hexosamine recovered (μg.)	Destruction of added glucosamine (%)
Glucosamine hydrochloride	4	4*	10·0	—	8·75	12·5
Bovine synovial fluid	4	4*	31·5	30·0	54·0	12·2
Sheep gastric mucin	4	4*	3·0	5·0	7·0	12·5
Hyaluronic acid complex	4	4*	22·5	20·0	38·5	9·5
Glucosamine hydrochloride	4	12	10·0	—	9·4	6·0
Hyaluronic acid (umbilical cord)	4	12	73·5	70·0	133·5	7·0

* Hydrolysis in sealed tubes.

with the scale, such as the surface : volume ratio of the vessels. The smaller losses found in the stoppered tubes may have been due to these not being wholly immersed. The excess of acid after hydrolysis was removed by evaporation to dryness *in vacuo* over potassium hydroxide to prevent breakdown of liberated hexosamine (Kent & Whitehouse, 1955).

Effect of resin separation

Several workers (Vasseur & Immers, 1949; Horowitz, Ikawa & Fling, 1950) have observed that the presence of reducing sugars and amino acids causes considerable error in the estimation of hexosamine by the method of Elson & Morgan. Experiments conducted in this laboratory showed that the method of Dische & Borenfreund (1950) suffered from the same defect. The only specific method described for removing these interfering substances is that devised by Boas (1953), who separated the sugars on a Dowex 50 column. Column exchange on an ultramicro scale was found impracticable, but it was possible to remove quantitatively small quantities of hexosamine from hydrolysates containing sugars and amino acids by shaking with a few grains of Amberlite IRC 50 resin buffered at pH 7. The pH of the solution was maintained below the isoelectric points of the amino sugars and above that of most of the amino acids, with the exception of arginine and lysine, which are absorbed. These have been shown not to interfere in the reaction with pyrrole. Estimating 5·0 μg. of glucosamine hydrochloride by the micro modification of the pyrrole method (total volume, 6 ml.) gave an extinction coefficient ($E_{1\,cm.}$) at 433 mμ of 0·15. In the presence of 500 μg. of histidine hydrochloride $E_{1\,cm.}$ was 0·16; in the presence of a similar amount of arginine hydrochloride $E_{1\,cm.}$ was 0·14, and with lysine $E_{1\,cm.}$ was 0·15.

A series of determinations with glucosamine standards was made with and without resin separation (Table 2). The results did not depart significantly from linearity with quantity. The recovery was 96·7 ± 0·4 % over the whole range. The results obtained by treatment with resin should therefore be multiplied by 1·03. A number of biological materials were treated with resin (Table 3). Synovial fluid and guinea-pig serum gave values 10–15 % lower after resin treatment. The differences with *Mycobacterium tuberculosis* polysaccharide and hyaluronic acid complex were smaller but consistent. These results suggest that, as previously suspected (Kent & Whitehouse, 1955), hitherto available methods for estimating hexosamine in serum and synovial fluid have given high values due to interferences from sugars and amino acids.

Table 2. *Recovery of glucosamine before and after absorption and elution from buffered* (pH 7) *Amberlite* IRC-50 *resin*

Amounts varying between 0·085 and 0·42 μg. of glucosamine hydrochloride were estimated by the ultramicro pyrrole method before and after resin separation. Measurement was made in the Bessey & Lowry (1946) cells. Glucosamine contents were calculated as $C_6H_{13}O_5N$.

Glucosamine (μg.)	$E^{433\,m\mu}_{1\,cm.}$ (without resin)	$E^{433\,m\mu}_{1\,cm.}$ (with resin)
0·070	0·083	0·080
	0·087	0·085
	0·085	0·0825
	0·0875	0·085
0·100	0·130	0·125
	0·130	0·123
	0·130	0·125
	0·130	0·123
0·140	0·175	0·170
	0·175	0·170
0·280	0·330	0·335
0·375	0·490	0·475

Table 3. *Estimation of hexosamine from hydrolysates of biological materials before and after treatment with resin*

The fluid (100 μl.) was treated with or without resin and the hexosamine content estimated by the micro pyrrole method. Values after resin treatment have been multiplied by 1·03 (see text). The sources of the materials are given in Table 6. Where no concentration is given the fluid was at natural biological concentration, as received. The hexosamine was calculated as $C_6H_{13}O_5N$ and corrected for measured loss during acid hydrolysis.

Material	Conditions of hydrolysis: HCl concn. (N)	Conditions of hydrolysis: Time (hr.)	Concn. of test substance (mg./100 ml.)	Hexosamine content (μg./ml.): Without resin (*A*)	Hexosamine content (μg./ml.): With resin (*B*)	Recovery after resin treatment (100 *B*/*A*)
Bovine synovial fluid	4	4*	—	280	249	88·8
Sheep gastric mucin	4	12	100	30	30	100·0
Hog gastric mucin (group A)	2	2	300	690	693	100·4
Hog gastric mucin (group O)	2	2	300	140	139	99·3
Hyaluronic acid (umbilical cord)	4	4*	300	600	600	100·0
Guinea-pig serum	2	12	—	830	703	84·7
Hyaluronic acid complex	4	4*	—	220	217	98·6
Myco. tuberculosis polysaccharide (human strain) $[\alpha]_D + 25°$	2	2	300	125	123·5	99·0
Glucosamine hydrochloride	—	—	10	85	85·3	100·3

* Hydrolysed in sealed tubes.

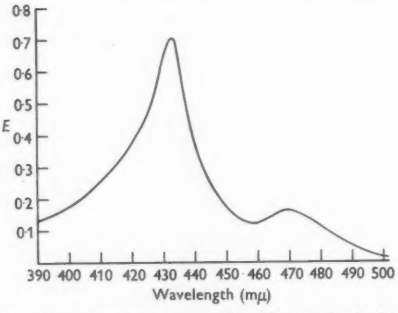

Fig. 2. Absorption spectrum produced by treating hydroxymethyldihydroxytetrahydrofurfuraldehydes with HCl and pyrrole as described in the text.

Colour development

Reaction of hydroxymethyldihydroxytetrahydrofurfuraldehydes with dilute hydrochloric acid and indole develops an orange colour which was compared with the colour produced with indole before deamination (Dische & Borenfreund, 1950). Increase of acid concentration from 0·25 to 0·75 N increased the sensitivity, possibly due to an increase in hydroxymethylfurfuraldehyde or δ-hydroxylaevulic aldehyde. One drawback, however, was that indole gave heavy precipitates and very high blanks with concentrations of acid greater than 0·5 N. It was found that stannous chloride inhibited high blank formation but that the sensitivity was reduced. Of the many chromogenic reagents tried the most sensitive found was pyrrole. Pyrrole produces a yellow colour with hydroxymethyldihydroxytetrahydrofurfuraldehydes and hydrochloric acid with a characteristic absorption curve (Fig. 2). The maximum was 433 mμ, with a small peak at 468 mμ. The $\epsilon_{433\,m\mu}$ of glucosamine was $4{\cdot}3 \times 10^4$. Galactosamine gives the same result. Reaction with pyrrole is sensitive to temperature and time of heating (Figs. 3 and 4), so that blanks and standards must be treated in exactly the same manner. When kept cool (below 10°) the colour is stable for 2–3 hr. after development.

The final conditions chosen (heating for 5 min. at $60 \pm 1°$ in 6·75 N-hydrochloric acid) gave maximum sensitivity and minimum blank.

Accuracy and sensitivity

The accuracy of the new method was tested by making a series of determinations with glucosamine standards (Table 4). Statistical analysis of these results show that there are no significant differences between the standard deviations of these different sets and that there is no systematic change of standard deviation with amount of hexosamine estimated. The standard error of a single estimation, taken from all the results in Table 4, is 0·0157 of extinction, equivalent to $\pm 2{\cdot}55$ μmg. of hexosamine. Fig. 5 shows that the difference between test solution and blank is linear with amount of hexosamine from 11 to 110 μmg., a value of $E_{5\,cm.}$ of 0·1 corresponding to 16·15 μmg. of hexosamine. The error is less than $\pm 6\%$ for duplicate estimations of amounts greater than 30 μmg.; sextuplicate estimations will give an error of $\pm 7\%$ on 10 μmg. The pyrrole reaction is four times more sensitive than any other, and six times more sensitive than that of Dische & Borenfreund (1950) in terms of measured

extinction/wt. of glucosamine. It can be scaled down to estimate 1000 times less material than can be estimated by any method previously described.

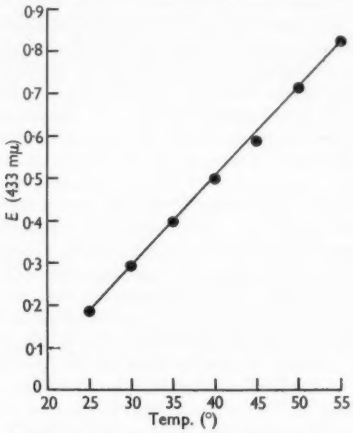

Fig. 3. Effect of temperature on colour production by glucosamine hydrochloride (22·5 μg.). Deaminated glucosamine (1 ml.) was treated with 3 ml. of conc. HCl and 10 μl. of a 1% solution of pyrrole in methanol. No stannous chloride was present. The solution was heated for 5 min. at various temperatures. The volume was made up to 6 ml. and colours were read in 1 cm. cells. Colours were read against blanks treated in a similar manner.

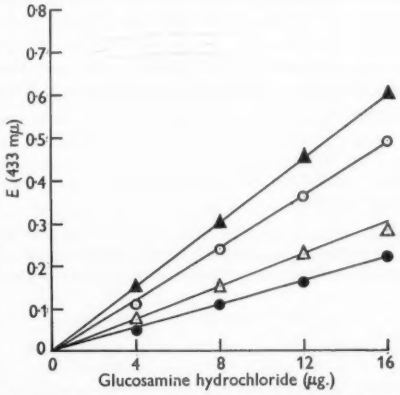

Fig. 4. Effect of time of heating on the pyrrole colour reaction. The varying amounts of glucosamine hydrochloride were deaminated and diluted to 1 ml. Conc. HCl (3 ml.) and 10 μl. of a 1% solution of pyrrole in methanol were added. No stannous chloride was present. Colours were read in 1 cm. cells. Total volume was 4 ml. Determinations were made against blanks treated in a similar manner. The solutions were heated at 45°. ●, 2·0 min.; △, 2·5 min.; ○, 4·0 min.; ▲, 5·0 min.

Ultramicro modification of the method of Elson & Morgan

Tests with known amounts of glucosamine standards by the ultramicro version of the method of Elson & Morgan showed that the present modifications increased the sensitivity about 1000-fold, and enabled 30–300 μmg. of hexosamine to be estimated (Fig. 6). This was brought about by scaling down the quantities to ultramicro level and by increasing the alkalinity of the acetylacetone–sodium carbonate reagent. Fresh reagent must be prepared for each series of determinations, because under these strong alkaline conditions acetylacetone is not stable.

Table 4. *Calibration of the pyrrole reagent with glucosamine hydrochloride*

Glucosamine hydrochloride standards were determined by the ultramicro pyrrole method as described. The blank was read against water; the rest of the determinations were read against the blank. Glucosamine contents were calculated as $C_6H_{13}O_5N$.

Glucosamine (μmg.)	No. of determinations	$E^{433\,m\mu}_{5\,cm.}$ (mean ± S.D.)
None (blank)	15	0·185 ± 0·0146
11	6	0·089 ± 0·0124
22	12	0·140 ± 0·0120
33	6	0·213 ± 0·0116
44	6	0·273 ± 0·0163
55	12	0·340 ± 0·0162
66	6	0·404 ± 0·0246
88	6	0·557 ± 0·0137
110	6	0·668 ± 0·0183

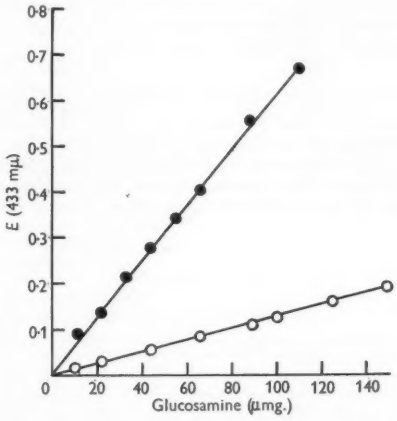

Fig. 5. Calibration graph with glucosamine standards, the ultramicro pyrrole method being used. ○, Bessey & Lowry cells; ●, capillary cells.

Comparison of methods

Results obtained in the past by comparing the method of Dische & Borenfreund with the method of Elson & Morgan have shown large discrepancies. Dische (1955) reported results 45% lower and McCrea (1951) 20% higher than the Elson & Morgan values for the hexosamine content of acid mucopolysaccharides. Other high results have been reported in the presence of sugars and amino acids (Kent & Whitehouse, 1955). This suggests that the material was not completely hydrolysed in the procedure of Dische & Borenfreund, whereas the high results reported by McCrea suggest interferences from sugars and amino acids. The results in Table 5 show that without resin separation interferences from sugars and amino acids affect both the Elson & Morgan and pyrrole procedures to about the same extent. That reproducible values are obtained by both methods suggests that the materials were completely hydrolysed. If the acidic hydrolysis was incomplete, the results without resin separation would be expected to differ because the acidic deamination and colorimetric conditions of the pyrrole and Dische & Borenfreund methods may cause further deacetylation, or breakdown of glycosyl linkages to occur to a different degree from the alkaline conditions of the Elson & Morgan method. Agreement between the two methods after resin separations (Table 5) was achieved as expected since only free hexosamine is separated by the resin and interference is eliminated. Comparison was also made between the ultramicro pyrrole procedure, with 0·1–1·0 μl. samples, and the Elson & Morgan micro method, with 100 μl. samples, for a range of biological materials (Table 6). Agreement was obtained, as can be seen by the comparison made in the final column of this table. The values obtained in Tables 3, 5 and 6 are not absolute values. The materials were not necessarily pure; they were used merely to obtain a comparison between the methods.

The new method, besides being more sensitive, has the advantage of being more specific than previous methods, and laborious column exchange is not used. The method is not critically affected by small differences in pH and heating conditions, as is the Elson & Morgan procedure, and it appears that only one chromogen is formed instead of three or four found in the latter (Schloss, 1951), and the reagent blank is low. By increasing the volume of the reagents 30-fold the method has been readily adapted to the usual micro scale when 1–10 μg. of hexosamine can be determined.

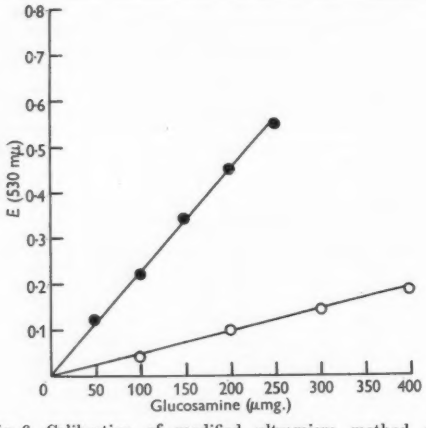

Fig. 6. Calibration of modified ultramicro method of Elson & Morgan with glucosamine standards. ○, Bessey & Lowry cells; ●, capillary cells.

Table 5. *Comparison of pyrrole and the Elson & Morgan* (1933) *procedures with and without resin separation*

Hexosamine in the biological materials below was estimated by the micro pyrrole procedure and the Elson & Morgan micro method with and without resin separation. Except for guinea-pig serum, the solutions had the same concentrations as in Table 3. The correction factor of 1·03 was made after resin treatment (see text). The hexosamine was calculated as $C_6H_{13}O_5N$ and corrected for measured loss during acid hydrolysis.

	Conditions of hydrolysis		Hexosamine content (μg./ml.)			
			Elson & Morgan method		Pyrrole method	
Material	HCl concn. (N)	Time (hr.)	Without resin	With resin	Without resin	With resin
Hyaluronic acid complex	4	4*	190	190	212·5	210
Bovine synovial fluid	4	4*	270	240	272·5	250
Guinea-pig serum	2	12	590	500	562·5	490
Hog gastric mucin (group A)	2	2	670	660	675	650
Hog gastric mucin (group O)	2	2	125	122	130	127
Hyaluronic acid (umbilical cord)	4	4	590	600	575	570
Glucosamine hydrochloride	—	—	85	85	83	85

* Hydrolysed in sealed tubes.

Table 6. *Comparison of single hexosamine determinations by the Elson & Morgan micro method and the ultramicro pyrrole method*

The micro method of Elson & Morgan was compared with the ultramicro pyrrole method as described in the text. Ultramicro samples ranged from 0·1 to 1·0 μl. and contained between 14 and 108 μmg. of hexosamine. The sample volumes were measured with an ultramicro burette. The micro samples ranged from 100 to 200 μl. in volume, containing between 14 and 108 μg. Where no concentration is given the fluid was at natural concentration as received. Hexosamine was calculated as $C_6H_{13}O_5N$ and corrected for measured loss during acid hydrolysis. Except for guinea-pig serum, hyaluronic acid complexes II and III and hyaluronic acid (umbilical cord), the samples were the same as in Tables 3 and 5.

Material examined	Concn. (mg./100 ml.)	Conditions of hydrolysis: HCl concn. (N)	Conditions of hydrolysis: Time (hr.)	Mean hexosamine contents of sample (μmg./μl.): Micro	Mean hexosamine contents of sample (μmg./μl.): Ultramicro
Hyaluronic acid (umbilical cord)[1]	300	4	4†	450	432
Glucosamine hydrochloride	9	—	—	77·5	75
Hyaluronic acid complex (sample I)	—	4	4†	220	220
Hyaluronic acid complex I + glucosamine hydrochloride	10*	4	4†	149‡	150‡
Hyaluronic acid complex (sample II)	—	4	4†	295	300
Hyaluronic acid complex II + glucosamine hydrochloride	10*	4	4†	187‡	180‡
Hyaluronic acid complex (sample III)	—	4	4†	180	184
Hyaluronic acid complex III + glucosamine hydrochloride	10*	4	4†	129‡	128‡
Human serum	—	2	12	845	880
Guinea-pig serum	—	2	12	945	880
Human cerebrospinal fluid	—	2	12	10	14
Bovine synovial fluid	—	4	12	265	276
Sheep gastric mucin[3]	100	4	12	24	25
Hog gastric mucin (group A)[4]	300	2	2	725	650
Hog gastric mucin (group O)[4]	300	2	2	130	116
Myco. tuberculosis (human strain)[5] $[\alpha]_D + 25°$	300	2	2	115	110
Heparin (B.D.H. specimen)	100	4	12	205	200

* Concentration of glucosamine hydrochloride mixed with complex. All the three samples of complex were made by the same technique (reference 2) but their concentrations were different.

† Hydrolysed in sealed tubes.

‡ Hexosamine content of mixed samples.

[1] McClean (1943); [2] Ogston & Stanier (1950); [3] Kent, P. W., Pasternak, C. & Davies, R. E. (personal communication); [4] Morgan & King (1943); [5] Haworth, Kent & Stacey (1948).

SUMMARY

1. An ultramicro method is described for the determination of hexosamine. The hexosamine is separated from other sugars by shaking with an ion-exchange resin, deaminated and treated with pyrrole and hydrochloric acid to produce a yellow colour having $E_{max.}$ at 433 mμ and $\epsilon_{433\ m\mu}$ of hexosamine = $4{\cdot}3 \times 10^4$.

2. The method is applicable to a range 10–100 μmg., which can be determined with an overall accuracy of ± 5 % for duplicate samples. The basic reaction is six times more sensitive than the Dische & Borenfreund (1950) reaction, and three times more sensitive than the most sensitive modification of the Elson & Morgan (1933) method. Scaling down the quantities to ultramicro level raises the sensitivity over previous methods to 1000-fold. It is specific for hexosamine.

3. The method has been used in comparison with an ultramicro modification of the method of Elson & Morgan to determine the hexosamine content of less than 1 μl. amounts of biological fluids. Excellent agreement between the two methods was found, and the significance of this is discussed with regard to complete hydrolysis and interference from sugars and amino acids.

4. The method is readily applicable to the usual micro scale (1–10 μg.), when conventional apparatus can be used.

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South African Pilchard Oil

6. THE ISOLATION AND STRUCTURE OF A DOCOSAHEXAENOIC ACID FROM SOUTH AFRICAN PILCHARD OIL*

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(*Received* 25 *February* 1957)

Docosahexaenoic acid has been reported many times in marine oils. Toyama & Tsuchiya (1935) isolated such an acid from sardine oil, and suggested it was either the 4:8:12:15:18:21- or the 4:8:11:14:17:20-hexaene. Farmer & Van den Heuvel (1938) isolated it from cod-liver oil, and gave five possible structures, all with the end double bonds in positions 4 and 20. Matsuda (1942) obtained a docosahexaenoic acid from bonito oil, and proposed a $\Delta^{4:8:12:15:18:21}$ structure for it. Notevarp, Roald, Sletness & Nordli (1953) have reported the isolation of docosahexaenoic acid from herring oil, while Abu-Nasr & Holman (1954) obtained it from cod-liver oil and determined some of the properties of the ethyl ester.

Docosahexaenoic acids have also been obtained from other natural sources. In each the 4:7:10:13:16:19-hexaene structure has been proposed. Klenk & Lindlar (1955) isolated one from the glycerophosphatides of ox brain, and Klenk & Dreike (1955) have reported the presence of the acid in ox-liver phospholipid. A similar acid was isolated by Hammond & Lundberg (1953*b*) from hog-brain lipids. These authors inferred the structure on biogenetic grounds and from alkali-isomerization evidence.

The isolation of a docosahexaenoic acid from South African pilchard oil (*Sardina ocellata* Jenyns) is now reported. The methods of separation and identification were similar to those described in earlier papers of this series (see Silk & Hahn, 1954*b*; Whitcutt & Sutton, 1956). The main product is considered to be the all-*cis* form of n-*docosa*-4:7:10:13:16:19-*hexaenoic acid*. The acid is believed to be between 84 and 90% pure.

* Part 5: Whitcutt & Sutton (1956).
† Marine Oils Research Fellow.

EXPERIMENTAL AND RESULTS

Melting points are uncorrected. General methods have been described in a previous paper (Whitcutt & Sutton, 1956). Ultraviolet spectra were determined on a Unicam model SP. 500 spectrophotometer and infrared spectra on a Perkin–Elmer model 21 double-beam infrared spectrophotometer.

Isolation of the docosahexaenoic acid

Preparation of an unsaturated acid concentrate. Total pilchard-oil acids (967 g.) were fractionated by means of the lithium salt–acetone and urea-complex procedures to give 254 g. of unsaturated acid concentrate (equiv. wt., 310; iodine value, 395). The procedures were the same as those described by Silk, Sephton & Hahn (1954), except that the precipitation of the acids forming urea complexes was carried out in a single stage.

Esterification of the acid concentrate. The concentrate (232 g.) was dissolved in methanol (1 l.), boiled at 60 mm. pressure for ½ hr. with Norit F.Q.P. decolorizing charcoal (20 g.), filtered and allowed to stand for several hours at 0° in the presence of excess of diazomethane in ether. After removal of diazomethane and some ether, the product was separated between pentane and 10% aq. Na_2CO_3. Methyl esters (232 g.) were recovered from the pentane layer.

Molecular distillation of the unsaturated esters. The ester concentrate was subjected to three-stage molecular distillation in the still described by Sutton (1953), the temperature and the rate of flow being adjusted so that about one-third of the material was removed as distillate after each cycle of three distillations. The fractions shown in Table 1 were obtained.

Distillate 4 (Table 1) was mixed with 100 ml. of 8% methanolic KOH at −70° and allowed to stand with occasional shaking for 4 hr. at 30° under vacuum. Water (100 ml.) was then added and the solution extracted twice with pentane to remove unchanged ester. After acidification with HCl and further extraction with pentane, 11·5 g. of a clear yellow oil was obtained. This material (distillate-4 acids) had equiv. wt., 333; iodine value, 424.

Reversed-phase partition chromatography of distillate-4 acids. The conditions were the same as those described by Whitcutt & Sutton (1956). The graph obtained when portions of the column eluate were titrated with methanolic 0·01 N-KOH is shown in Fig. 1. The acids were recovered by diluting each fraction with an equal volume of water and extracting with pentane (see Silk & Hahn, 1954*b*). In all, about 500 mg. of fraction II was recovered from six chromatograms. Corresponding samples were not mixed but were assumed to be identical.

Fraction I was found to consist of the eicosapentaenoic acid described by Whitcutt & Sutton (1956).

Properties and structure of the docosahexaenoic acid

General properties. The *docosahexaenoic acid* recovered from fraction II was a clear, faintly yellow oil of m.p. −44·5 to −44·1°, refractive index n_D^{26} 1·5017 [Found: C, 80·5, H, 9·7%. No. of double bonds/mol., 5·96 (hydrogen uptake over Pd–$BaSO_4$); equiv. wt., 324; iodine value, 446. $C_{22}H_{32}O_2$ requires C, 80·4; H, 9·8%. No. of double bonds/mol., 6·00; equiv. wt., 328; iodine value, 464.]

Table 1. *Molecular distillation of the unsaturated ester concentrate at 96°*

Distillate	Wt. (g.)
1	135
2	30
3	17
4	13
Residue	30

A Kuhn–Roth determination (Eisenbraun, McElvain & Aycock, 1954) gave the number of *C*-Me groups/mol. as 1·02.

Hydrogenation product. Chain-length analysis of the above hydrogenation products (Silk & Hahn, 1954*a*) showed the presence of only C_{22} saturated acid.

The unsaturated acid (60 mg.) was hydrogenated over Pd–$BaSO_4$ in acetic acid. The product was filtered, extracted with pentane and crystallized twice from acetic acid. This material was used in the melting-point determinations below. The residues were recrystallized from acetone and used in all other determinations.

The saturated acid had equiv. wt. 342, m.p. 79·0–79·3°, compared with equiv. wt. 340, m.p. 79·95° for *n*-docosanoic acid (Francis & Piper, 1939). Mixed with synthetic *n*-docosanoic acid (m.p. 79·3–79·9°), it had m.p. 79·0–79·5°.

The X-ray long spacing of the product was 48·2 Å compared with 48·0 Å for synthetic *n*-docosanoic acid. Francis & Piper (1939) give a value of 48·3 Å for the *C* modification of *n*-docosanoic acid.

The X-ray powder patterns and infrared spectra in carbon disulphide of the hydrogenation product and of our synthetic acid showed no significant differences.

Oxidation products. The conditions of oxidation were similar to those described by Whitcutt & Sutton (1956). The unsaturated acid (30 mg.) was dissolved in dry ethyl acetate (5 ml.) and acetic anhydride (1 ml.) and cooled to −80°. Ozonized oxygen (3%) was passed through the solution at 120 ml./min. until a faint blue colour appeared. A stream of nitrogen was then blown through for 45 sec., after which acetic anhydride (1 ml.), H_2O_2 (1 ml. of '130 vol.') and acetone (3 ml.) were added with shaking. The solution stood overnight at 30° and was then boiled for 30 sec. and most of the solvent removed under vacuum. Potassium hydroxide solution (3 ml. of 25%, w/v) was added, and the product

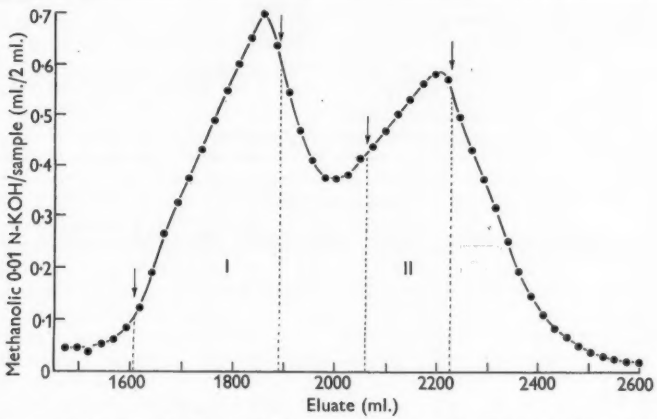

Fig. 1. Chromatogram of acids from distillate 4 (Table 1). Weight of sample, 500 mg. Column, heptane supported on non-wetting kieselguhr, 280 cm. × 1·5 cm. Temperature, 10°. Flow rate, 100 ml./hr. Solvent system: aq. methanol 70% (v/v) to 415 ml. of eluate, 70% (v/v) to 1200 ml., 75% (v/v) to 1265 ml., 80% (v/v) to 2600 ml. Solvents were equilibrated with *n*-heptane. Acetic acid (6 ml./l.) was added to the first solvent. Eluates between the arrows were collected separately as fractions I and II.

heated on a water bath for a few minutes, cooled, and acidified with a slight excess of conc. HCl. The product was evaporated to dryness under vacuum, and the residue extracted with boiling acetone (about 40 ml. in all). The dicarboxylic acids obtained by evaporation of the acetone were chromatographed on paper with chloroform–formic acid–water–acetone solvent as described by Whitcutt & Sutton (1956). Only malonic and succinic acids were identified.

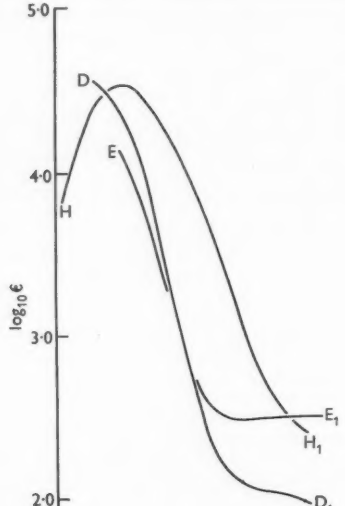

Fig. 2. Ultraviolet spectra of fatty acids in the region 200–240 mμ. HH_1, Heptadeca-2-enoic acid from Lauer, Gensler & Miller (1941); EE_1, eicosapentaenoic acid from Whitcutt & Sutton (1956); DD_1, docosahexaenoic acid isolated from pilchard oil.

A further sample of the unsaturated acid (17 mg.) was oxidized with CrO_3 under the conditions described by Eisenbraun *et al.* (1954), and the steam distillate concentrated to a small volume. Paper chromatography (Lindquist & Störgards, 1953) showed that the only volatile oxidation products were acetic and propionic acids.

Ultraviolet absorption. Apart from a slight inflexion at 232 mμ corresponding to 0·2% autoxidation (Silk & Hahn, 1954*b*), the docosahexaenoic acid showed no absorption in the 220–300 mμ region. The spectrum is given in Fig. 2.

Alkali isomerization. The docosahexaenoic acid (11·2 mg.) was heated at 180° under nitrogen in a 21% (w/w) solution of KOH in ethylene glycol for 8 min. (see Herb & Riemenschneider, 1952; Hammond & Lundberg, 1953*a*). The absorption spectrum is shown in Fig. 3.

Infrared absorption. The spectrum shown in Fig. 4 was taken in carbon tetrachloride solution (1·30%, w/w) except from 850 to 670 cm.$^{-1}$, where carbon disulphide solution (1·26%, w/w) was used.

Synthesis and degradation of methyl tricosahexaenoate. The docosahexaenoic acid (41 mg.) was used to prepare the methyl ester of its higher homologue (see Whitcutt & Sutton, 1956). The product was ozonized, worked up as described above and the dicarboxylic acid fragments were chromatographed on paper. Malonic and glutaric acids were detected together with a smaller amount of succinic acid. The ozonolysis was repeated with the following modifications.

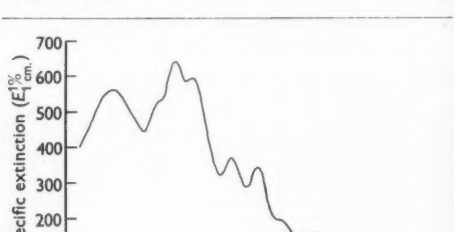

Fig. 3. Ultraviolet absorption of docosahexaenoic acid after alkali isomerization. For details see text.

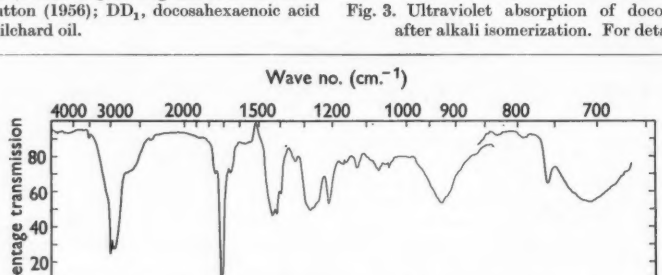

Fig. 4. Infrared absorption of docosahexaenoic acid. For details see text. The band at 759 cm.$^{-1}$ may be due to traces of chloroform.

After blowing nitrogen through the ozonized solution, H_2O_2 (0·4 ml. of '130 vol.') was added, together with enough acetone (about 3 ml.) to give one phase. This solution was allowed to stand for 20 min. at room temperature before being boiled for a few seconds. Most of the solvent was then removed under vacuum. Water (3 ml.) was added, and excess of solid Na_2SO_3 to reduce all the H_2O_2 present. After warming and then standing for a few minutes, KOH solution (4 ml. of 25 %, w/v) was added, and the procedure continued as described above. Only malonic and glutaric acids were detected when the final product was chromatographed on paper.

DISCUSSION

The unsaturated acid melted over a range of 0·4°. Its elementary analysis, equivalent weight and hydrogen uptake correspond to the values which would be expected for a docosahexaenoic acid.

Chromatographic examination of the hydrogenated material showed that only C_{22} acids were present. The hydrogenated counterpart, recrystallized from acetic acid, began to melt 0·9° below the upper limit of melting of our authentic specimen of *n*-docosanoic acid, and this latter point coincided with the literature value. A mixed melting point showed no depression. The X-ray long spacing of 48·2 Å is in agreement with the result (48·0 Å) on our authentic acid and with the value (48·3 Å) reported by Francis & Piper (1939). Neither the X-ray-powder pattern nor the infrared spectrum differed significantly from those of *n*-docosanoic acid.

The iodine value (446) is the only analytical result appreciably different from the theoretical value (464) for docosahexaenoic acid, but the difference is too small to be accounted for by the presence of acetylenic or allenic unsaturation. To settle the question of the exact degree of unsaturation of the acid our microhydrogenation procedure was tested with pure samples of unsaturated acids. Results were found to be reproducibly 1–2 % high, and on this basis the correct unsaturation of the acid is 5·84–5·90 double bonds/molecule. Assuming, as is most probable from the known chromatographic behaviour of these compounds, that docosapentaenoic acid is the main contaminant, the docosahexaenoic acid is between 84 and 90 % pure.

Spectral evidence shows that the double bonds are not conjugated with each other nor with the carboxyl group, since there are no bands in the ultraviolet, and absorption below 240 mμ is much less than that shown by *n*-heptadec-2-enoic acid. The absence of infrared bands at 968, 990 and 3300 cm.$^{-1}$ rules out the possibility of *trans* or terminal unsaturation (see Bellamy, 1954). A broad absorption band between 1370 and 1500 cm.$^{-1}$ may have obscured the characteristic 1379 cm.$^{-1}$ band of the methyl group. A distinct shoulder possibly due to the latter appears at 1395 cm.$^{-1}$.

The only oxidation products detected by paper chromatography were acetic, propionic, malonic and succinic acids. Acetic acid may have arisen from further oxidation of propionic acid; both acids were present to approximately the same extent. Malonic acid was by far the most abundant product.

The advantages of using an Arndt–Eistert synthesis to determine the position of the double bond nearest to the carboxyl group have been previously discussed (Whitcutt & Sutton, 1956). Ozonolysis of the C_{23} homologue of the docosahexaenoic acid gave only malonic and glutaric acids.

On the basis of the evidence outlined above the main component of the material isolated can only be $H_3C{\cdot}CH_2{\cdot}[CH{:}CH{\cdot}CH_2]_6{\cdot}CH_2{\cdot}CO_2H$ (all *cis*-*n*-docosa-4:7:10:13:16:19-hexaenoic acid). However, the possibility that other docosahexaenoic acids with different structures are also present in smaller amounts cannot be excluded.

The alkali-isomerization spectrum of the acid shows bands corresponding to conjugated diene, triene, tetraene, pentaene and hexaene, and is supporting evidence for the group

$$\cdot[CH{:}CH{\cdot}CH_2]_5{\cdot}CH{:}CH\cdot,$$

since de Surville, Sutton & Rivett (1957) have shown that the doubly methylene-interrupted double bonds of trideca-5:9-dienoic acid do not isomerize into conjugation under the usual conditions for alkali rearrangement. The low proportion of conjugated pentaene and hexaene obtained in the isomerization of the docosahexaenoic acid contrasts with the results reported by other authors (see Hammond & Lundberg, 1953*a*; Abu-Nasr & Holman, 1954).

Since the structure proposed above is in disagreement with all the earlier structures for docosahexaenoic acids of marine origin, it is desirable to review the evidence upon which these structures were based. Although the acids were obtained from various sources, the fatty acid compositions of different fish oils are often similar with regard to the distribution of chain length and unsaturation, and it is reasonable to assume that the same acids are present.

Toyama & Tsuchiya (1935) proposed a $\Delta^{4:8:12:15:18:21}$, or alternatively a $\Delta^{4:8:11:14:17:20}$, structure, on the basis of a quantitative examination of oxidation products, but it is difficult to assess the value of their results as only minor amounts of pure products appear to have been isolated.

Matsuda (1942) proposed a $\Delta^{4:8:12:15:18:21}$ structure based in part on identification of the ozonolysis products after partial hydrogenation of the acid in the presence of a nickel catalyst. Boelhouwer, Gerkens, Ong Tian Lie & Waterman (1953), however, have shown that double bonds migrate readily under such conditions.

Farmer & Van den Heuvel (1938) based their alternative structures, all with double bonds in positions 4 and 20 and with a

$$\cdot CH{:}CH \cdot CH_2 \cdot CH_2 \cdot CH{:}CH \cdot$$

group somewhere in the chain, upon (i) identification of acetaldehyde, acetic acid and carbon dioxide as the only volatile oxidation products; (ii) identification of methyl hydrogen succinate after oxidation of the ester; (iii) production of 4·8 mol.prop. of acetic and 1·3 mol.prop. of succinic acid on complete oxidation of the free acid. It would be desirable to confirm these results by more modern techniques.

In no single case has the purity of a highly unsaturated C_{22} fatty acid of natural origin been properly established. Possible contaminants are closely related structures having similar chemical, physical and analytical properties which may counterbalance the effects produced by each other. Some polyene acids described in the literature, and with analytical values close to the theoretical ones, must to-day be considered of doubtful purity because of the relatively inefficient methods used in their isolation.

When closely related compounds are found together in Nature, biogenetic relationships may be of value in deciding the structure to be assigned in uncertain cases. The structure which has been proposed for the docosahexaenoic acid of South African pilchard oil is not only identical with that for the mammalian docosahexaenoic acids, but is also closely similar to the structure of the eicosapentaenoic acid described in Part 5 (Whitcutt & Sutton, 1956).

SUMMARY

1. Docosahexaenoic acid has been isolated from South African pilchard oil. Some of its properties have been determined and its degree of purity is discussed. Evidence is presented that the main component is the 4:7:10:13:16:19-hexaene.

2. The structures previously assigned to docosahexaenoic acids of marine origin are discussed.

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The Fluorimetric Estimation of Adrenaline and Noradrenaline in Plasma

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A fluorimetric method for the estimation of adrenaline and noradrenaline, based on a condensation with ethylenediamine, was applied to the analysis of plasma by Weil-Malherbe & Bone (1952, 1953). The concentrations found with this method were closer to those expected on theoretical grounds than many found by previous investigators. The validity of the method for plasma was supported by the following observations: the reacting material was removed by amine oxidase at the same rate as adrenaline (Weil-Malherbe & Bone, 1952) and separated by paper chromatography into two components with the same R_F values and yielding derivatives with the same fluorescent properties as adrenaline and noradrenaline (Weil-Malherbe & Bone, 1954*a*); also, the ethylenediamine (ED) method and the fluorimetric method of Lund (1950) yielded very similar results (Bone, 1953; Weil-Malherbe & Bone, 1954*b*; Weil-Malherbe, 1955*b*).

The level of the reacting material in plasma was studied under different conditions, and swift and often dramatic changes were observed, such as might be expected of adrenaline. Thus adrenaline estimates dropped after the injection of a barbiturate and rose after the induction of a convulsion (Weil-Malherbe, 1955*a*; see also Weil-Malherbe, 1955*b*, for a summary of results). Other investigators who used the method or modifications of it found satisfactory correlations between blood levels of catecholamines and various manifestations of sympathetic activity (Miller & Elliott, 1954; Woods, Richardson, Richardson & Bozeman, 1956; Bollman, Maher & Manger, 1956). On the other hand, the abrupt decrease of plasma adrenaline found by us after injection of insulin (Weil-Malherbe & Bone, 1954*c*) was at variance with many well-established facts. Holzbauer & Vogt (1954), by biological methods, have since found a rise; they also found a much lower initial level of adrenaline than that indicated by the fluorimetric methods. These findings, and the development of some new techniques mentioned below, have led us to reinvestigate the specificity of the ED method, as applied to plasma.

The earlier plasma analyses, and among them those which showed agreement between the ED method and the method of Lund, were carried out on eluates from a column of aluminium oxide, an adsorbent which retains catechols indiscriminately. Catechol compounds other than adrenaline and noradrenaline form fluorescent compounds with ED; among these, 3:4-dihydroxyphenylacetic acid and 3-hydroxytyramine are present in relatively large amounts in urine (Euler, Euler & Floding, 1955; Holtz, Credner & Koepp, 1942; Euler, Hamberg & Hellner, 1951). They are not estimated by those fluorimetric methods which depend on the formation of 3:5:6-trihydroxyindoles (THI; Lund, 1950; Euler & Floding, 1955*a*). A method has been developed in which 3:4-dihydroxyphenylacetic acid and possibly other neutral and acid catechols are removed from urinary alumina eluates by cation exchange (Weil-Malherbe & Bone, 1957). Two types of resin were employed: (1) Zeo-Karb 225, a strongly acidic resin, retains amino acids as well as amines; these two classes of compounds can be separated satisfactorily by paper electrophoresis. By a combination of exchange on Zeo-Karb 225 with paper electrophoresis the occurrence of 3:4-dihydroxyphenylalanine (dopa) in a phaeochromocytoma has recently been demonstrated (Weil-Malherbe, 1956). (2) Amberlite IRC-50, a weakly acidic resin which retains amines only. It was of interest to apply these methods of purification to plasma.

Since hydroxytyramine forms a fluorescent derivative in the ED but not in the THI method, it is possible to analyse a mixture of adrenaline, noradrenaline and hydroxytyramine by a combination of both methods (Weil-Malherbe & Bone, 1957). In the present experiments therefore both methods were used on most samples. If the estimates for adrenaline and noradrenaline agree, the absence of hydroxytyramine may be inferred.

In our earlier paper-chromatographic experiments (Weil-Malherbe & Bone, 1954*a*) the presence of catechols other than adrenaline and noradrenaline has not been completely excluded. More conclusive results have now been obtained by using the purified basic fraction for paper chromatography and by analysing the extracts of paper sections by the combined fluorimetric methods.

Finally, extracts of plasma purified by resin treatment were used for bioassay. Results previously obtained were inconclusive owing to the presence of interfering substances. It was hoped that the new methods might reduce or abolish this interference.

METHODS

Preparation of crude plasma extracts

Bovine blood was collected at the slaughterhouse less than 2 min. after the animal had been stunned by a 'humane killer'; about 1600 ml. was run from the severed blood vessels of the neck into 400 ml. of an isotonic solution containing 1% (w/v) of sodium ethylenediaminetetra-acetate (EDTA), pH 7, and 2% (w/v) of $Na_2S_2O_3,5H_2O$ (Renton & Weil-Malherbe, 1956). Human blood was withdrawn from an antecubital vein with the aid of a transfusion needle mounted on a short length of polyvinyl tubing. About 160 ml. was run into 40 ml. of EDTA–$Na_2S_2O_3$ solution and samples from four donors were usually pooled for one experiment. Plasma, separated by centrifuging for 20 min. at 600 *g*, was diluted with an equal volume of 0·2 M-sodium acetate and the pH of the mixture was adjusted to 8·4 by the addition of 0·5 N-Na_2CO_3. Portions of 200–250 ml. were filtered through columns of 0·7 g. of alumina. Usually four to eight of these columns were set up side by side on a pressure manifold designed so that the pressure could be adjusted independently in each individual unit (Bone, 1953). Each column was prepared, washed and eluted as previously described (Weil-Malherbe & Bone, 1952).

Preliminary experiments had shown that, within the limits of the experiments, the concentration of catechol compounds in the eluate increased linearly with the volumes of plasma filtered through a column. The volume of plasma which could be filtered through a column was limited by its gradual clogging and by the need to finish this stage in a reasonably short time, rather than by its saturation.

Isolation of the basic fraction

Filtration through Zeo-Karb 225. Columns of 2 g. of moist Zeo-Karb 225 (water regain 1·8; 40–70 mesh, H^+ form; The Permutit Co., London, W. 4) were prepared as described by Weil-Malherbe & Bone (1957). When the volume of the eluate from the alumina columns was 50 ml. or less, one Zeo-Karb column was used; otherwise the eluate was divided between two columns. Each column was washed with 150 ml. of 0·2 M-acetic acid and eluted with 50 ml. of 0·2 M-$BaCl_2$ (Weil-Malherbe & Bone, 1957). Barium sulphate was precipitated by the addition of 2 ml. of 10 N-H_2SO_4 and centrifuged off.

Filtration through Amberlite IRC-50. The eluate from the alumina columns was adjusted to pH 6·5 and filtered through one or two columns of 2 g. of moist Amberlite IRC-50 (analytical grade, prepared and buffered according to Bergström & Hansson, 1951; British Drug Houses Ltd.); not more than 50 ml. was used per column. Each column was washed with 10 ml. of 0·2% (w/v) NaCl and eluted with 25 ml. of N-HCl, followed by 25 ml. of 0·01 N-HCl.

Further treatment of eluates from resin columns. For eluates from Zeo-Karb columns the procedure was usually as follows: the barium-free solution was brought to pH 3 by the addition of Dowex 2 (× 10, 50–100 mesh, OH^- form; The Dow Chemical Co., Midland, Mich.) after diluting it with 3 vol. of water; this was necessary to prevent losses. The filtrate was concentrated *in vacuo* to less than 0·5 ml. and the residue taken up in 5 ml. of acetone. Insoluble matter was discarded, the clear solution was again evaporated almost to dryness and the extraction with 5 ml. of acetone was repeated. For paper chromatography this acetone solution was concentrated to 1 ml.; for bioassay or electrophoresis it was evaporated to dryness and the residue was dissolved in 1 ml. of 0·01 N-HCl.

This procedure was time-consuming and often entailed considerable losses. Moreover, its application to eluates from Amberlite columns was complicated by a large inorganic residue after evaporation. The following procedure was therefore adopted for these eluates and also for Zeo-Karb eluates obtained in some of the later experiments: 0·1 vol. of 1% (w/v) EDTA and 0·02 vol. of a freshly prepared 2% (w/v) solution of ascorbic acid were added to the eluate and its pH was adjusted to 8·4. It was then filtered through a column of 0·7 g. of alumina. The column was washed as usual and eluted with 5 ml. of 0·1 N-H_2SO_4 followed by 5 ml. of water. A volume of 0·3 N-$Ba(OH)_2$ was added to the eluate to pH 3. After removal of the precipitate the solution was evaporated almost to dryness and the residue twice treated with acetone as described above.

Paper chromatography

One-dimensional chromatography was carried out on strips (48 cm. × 8 cm.) of Whatman paper no. 1, pretreated as described (Weil-Malherbe & Bone, 1954*a*). Two solvent systems were used: (1) *n*-butanol–acetic acid–water (4:1:5), organic phase (descending technique; approximate R_F values: noradrenaline, 0·28; adrenaline, 0·31; hydroxytyramine, 0·38); (2) phenol–water (3:1, w/v) (ascending technique; approximate R_F values: noradrenaline, 0·30; hydroxytyramine, 0·48; adrenaline, 0·52). A dish in the bottom of the jar contained 5 N-HCl saturated with phenol. Phenol was distilled over aluminium turnings immediately before use (Draper & Pollard, 1949).

The acetone solution of the plasma extract, which was made up to exactly 1 ml., was divided into three parts: 0·2 ml. was pipetted into 9·8 ml. of 0·01 N-HCl for fluorimetric analysis, 0·4 ml. was transferred to a paper strip in a line about 4 cm. long and the final 0·4 ml. was mixed with 0·1 ml. of an aqueous solution containing 1 μg. of adrenaline and 4 μg. of noradrenaline. This was transferred to a second paper strip and served as an internal standard. A paper blank and a guide strip were also included. The chromatograms were run at room temperature for 16 hr. Phenol chromatograms were washed in three changes of benzene purified by extraction with conc. H_2SO_4 and distillation.

The guide strip was sprayed with a mixture of ethylenediamine–10% (w/v) aq. NH_3 soln. (2:8) and dried in air. Catecholamines appeared as yellow fluorescent spots in u.v. light. The fluorescent zones were marked with pencil and transverse lines were drawn so as to effect optimum separation. The other three strips were cut across along superimposed lines. These sections, and others cut from the remaining areas of the papers, were extracted overnight with 10 ml. of 0·01 N-HCl and the extracts analysed by the two fluorimetric methods. Extracts of phenol chromatograms were shaken three times with freshly distilled, peroxide-free ether before analysis, to remove the last traces of phenol. The paper blanks were free from fluorescence.

Paper electrophoresis

An aq. plasma concentrate of 1 ml. was divided into three portions as for paper chromatography. A sample of 0·4 ml. and another one to which adrenaline and noradrenaline had been added were each applied, near the anode, to a strip

Table 1. *Concentration of adrenaline and noradrenaline in bovine and human plasma*

Estimations were made by the ethylenediamine method.

	No. of expts.	Adrenaline		Noradrenaline	
		Means ± S.E.	Range	Means ± S.E.	Range
		(μg./l. of plasma)			
Bovine	15	6·00 ± 1·16	2·44–12·6	14·54 ± 2·16	6·0–26·5
Human	9	1·09 ± 0·095	0·55–1·44	4·14 ± 0·39	2·58–6·48

Table 2. *Recoveries after passage of alumina eluates of plasma through cation-exchange resins*

Results are means ± S.E.M. expressed as adrenaline or noradrenaline estimated by the ethylenediamine (ED) method or by the trihydroxyindole (THI) method.

Source of plasma	No. of expts.	Resin	Percentage recovery			
			Adrenaline		Noradrenaline	
			ED	THI	ED	THI
Bovine	9	Zeo-Karb 225	75·4 ± 2·42	79·5 ± 4·07	80·0 ± 3·98	81·5 ± 3·23
Bovine	6	Amberlite IRC-50	97·6 ± 4·44	99·9 ± 2·80	90·7 ± 3·06	97·2 ± 3·06
Human	1	Zeo-Karb 225	88·5	—	107·5	—
Human	8	Amberlite IRC-50	96·1 ± 8·32	97·7 ± 3·54	87·8 ± 4·69	88·7 ± 5·84

(34 cm. × 6 cm.) of Whatman 3MM paper, pretreated in the same way as the paper used for chromatography. The EEL paper-electrophoresis apparatus (Evans Electroselenium Ltd., Harlow, Essex) was used, with 0·04 M-sodium acetate buffer, pH 5·6, and a potential gradient of about 10 v/cm. Under these conditions catecholamines migrated 8–10 cm. towards the cathode in 2 hr., while dopa remained near the anode. At the end of this period the paper strips were dried and cut into sections which were extracted and analysed as described above.

Bioassays

These were carried out on rat uterus and rat ascending colon, rhythmically stimulated with acetylcholine (Gaddum, 1950). The samples tested (0·05–0·1 ml.) were taken from aqueous concentrates of 1–5 ml.; sometimes they had to be diluted further.

Fluorimetric analysis

Samples were usually diluted to 10 ml.; 2 ml. was used for the ED method (Weil-Malherbe & Bone, 1952, 1953) and three portions each of 2 ml. for the THI method (Euler & Floding, 1955*a*). Details of the procedure followed for the THI method and recent modifications of the fluorimeter have been described by Weil-Malherbe & Bone (1957).

RESULTS

Concentrations of catecholamines in bovine and human plasma

The experiments with bovine blood were intended mainly as preparation for experiments with human blood. They are open to the objection that the blood was obtained when the animal was in a state of stress. Table 1 shows that the concentrations of adrenaline and noradrenaline were indeed higher and more variable than those found in human plasma. This might be expected if catecholamines were discharged in varying intensity while ox blood was collected.

Recovery from cation-exchange resins

The amounts of adrenaline and noradrenaline estimated in eluates from resin columns were expressed as percentages of those found before this treatment. The results are shown in Table 2. The recoveries are similar, whether the two kinds of blood, the two amines or the two methods of estimation are compared. They are a little higher, at least in the experiments with ox blood, after passage through Amberlite than after passage through Zeo-Karb. If the small losses found after Zeo-Karb treatment had been caused by the removal of neutral or acid components, which, as a rule, are not estimated in the THI method, the two methods would not have been equally affected. If dopa had been present, the recoveries with Amberlite would have been below those with Zeo-Karb, whereas the reverse was found. The losses after Zeo-Karb treatment therefore appear to be unspecific, probably due to irreversible adsorption.

Correlations between ethylenediamine and trihydroxyindole methods

In Table 3 the results obtained by the two fluorimetric methods at various stages of the procedure have been compared. Results by the THI method, expressed as percentages of the results by the ED method, do not significantly deviate from 100 in most cases. There is a tendency for the adrenaline values to be above, and for the

Table 3. *Correlation of results obtained by the ethylenediamine and trihydroxyindole methods in extracts of plasma*

Results obtained by the trihydroxyindole method are expressed as percentages of results obtained by the ethylenediamine method. Means ± S.E.M.

	Bovine plasma			Human plasma		
	No.	Adrenaline	Noradrenaline	No.	Adrenaline	Noradrenaline
After passage through alumina	15	101·4±7·38	88·2±3·56*	8	111·7±6·15	98·8±3·64
After passage through alumina and Zeo-Karb 225	9	99·1±5·94	93·9±5·82	—	—	—
After passage through alumina and Amberlite IRC-50	6	109·2±11·99	89·7±6·26	9	98·5±5·85	96·6±1·73
Acetone extract of residue	12	110·0±6·08	95·9±3·95	7	107·1±7·12	100·1±4·75
All results combined	42	104·8±4·53	91·9±2·25*	24	105·4±3·68	98·3±1·88
Correlation coefficient (r)	42	0·947	0·995	24	0·820	0·912
Significance of correlation (P)	—	<0·001	<0·001	—	<0·001	<0·001

Significance of differences between values of r

	t	P
Bovine against human		
Adrenaline	2·39	0·02
Noradrenaline	5·26	<0·001
Adrenaline against noradrenaline		
Bovine	5·12	<0·001
Human	1·24	0·25

* Significantly different from 100 at 1% level of probability.

noradrenaline values to be below, 100%. This is undoubtedly due to the fact that a small proportion of noradrenaline (about 4–5% according to Euler & Floding, 1955*a*) is oxidized at pH 3·5 and is therefore estimated as adrenaline in the THI method. No correction has been made for this in the present results. It is noteworthy that the correlation is similar for both kinds of blood and that it does not change with the process of purification. It is generally held that if the ratio of two characteristics remains constant during purification they belong to the same entity, and the conclusion therefore seems justified that identical substances are measured by the two fluorimetric methods.

The correlation coefficients calculated from the pooled values indicate strong and significant correlations. Differences between coefficients were tested by Fisher's z-transformation (1944) and some were found to be significant; thus the correlation between the results of the two methods appears to be stronger in the experiments with bovine than in those with human blood. This may be merely a consequence of the larger number of figures available for the former; or it may be due to the higher and more widely scattered concentrations of adrenaline and noradrenaline in bovine plasma.

Paper electrophoresis

Two experiments were carried out with extracts of ox plasma which had been passed through Zeo-Karb 225 and which would have contained dopa, if it had been present in plasma. The results were similar in both experiments and one of them is shown in Fig. 1. The pattern of distribution and the

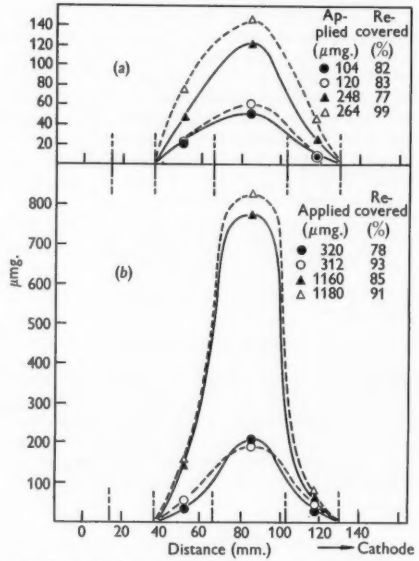

Fig. 1. Distribution of adrenaline and noradrenaline on paper strips after electrophoresis of bovine-plasma extract. △▲, Adrenaline; ○●, noradrenaline (filled-in points: estimated by the ethylenediamine method; open points: estimated by the trihydroxyphenol method). (*a*) Extract without addition; (*b*) internal standard after addition of adrenaline and noradrenaline. The markers at the abscissa indicate the dimensions of the paper sections analysed. Amounts applied and percentage recoveries are shown against the corresponding symbols.

recoveries of adrenaline and noradrenaline are very similar in the two paper strips and the two fluorimetric methods show similar agreement in both. There was no evidence for the presence of dopa; it would have remained near the line of application and would have been recognized by the development of fluorescence in the THI method, equal, under our conditions, to 12–14 % of the fluorescence of adrenaline and 35–40 % of that of noradrenaline when measured through a yellow secondary filter, and, further, by the formation of a fluorescent condensation product with ethylenediamine which is practically insoluble in *iso*butanol and therefore remains in the aqueous phase.

It is concluded from these experiments that the reactive material in plasma has the same electrophoretic mobility as adrenaline and noradrenaline.

Table 4. *Recoveries of adrenaline and noradrenaline from paper chromatograms of plasma extracts*

Where more than one experiment was performed the figures quoted are the means. Chromatogram I: extract without addition; chromatogram II: internal standard. A, Adrenaline; N, noradrenaline; ED, ethylenediamine method; THI, trihydroxyindole method.

Plasma	No. of expts.	Solvent	Chromatogram	Applied to paper (μmg.) A	Applied to paper (μmg.) N	Percentage recovery A By ED	Percentage recovery A By THI	Percentage recovery N By ED	Percentage recovery N By THI
Bovine	1	Butanol–acetic acid–water	I	405	1100	45	60	53	60
			II	1405	5100	43	48	63	52
Human	3	Phenol–water	I	107	317	82·3	81·3	98·6	91
			II	1107	4317	77	77	73	74·5
Human	2	Butanol–acetic acid–water	I	52	232	86·5	76·5	81	76·5
			II	1052	4232	84	69	73·5	72

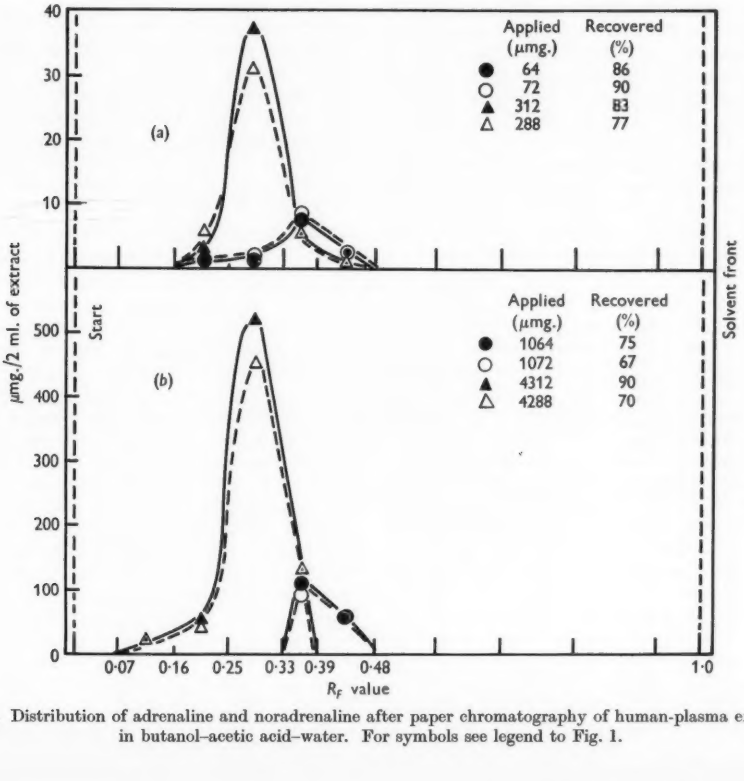

Fig. 2. Distribution of adrenaline and noradrenaline after paper chromatography of human-plasma extract in butanol–acetic acid–water. For symbols see legend to Fig. 1.

Paper chromatography

Table 4 shows the percentages of adrenaline and noradrenaline recovered after paper chromatography. These experiments were performed after purification by Amberlite IRC-50. Recoveries were similar for the preformed material and for internal standards and the correlation between the estimates obtained by the two fluorimetric methods was maintained after separation of the amines. These facts are illustrated in greater detail in Figs. 2 and 3, which show examples of butanol and phenol chromatograms of extracts of human plasma. The figures clearly demonstrate the similarity in distribution of adrenaline and noradrenaline in the samples with and without added amines. The agreement of results obtained on fractions separated by paper chromatography by both fluorimetric methods is further confirmed by five estimations of bovine-plasma extracts passed over Zeo-Karb 225; the means for adrenaline and noradrenaline (μmg./strip) were 140 and 567 for the ED method and 184 and 483 for the THI method.

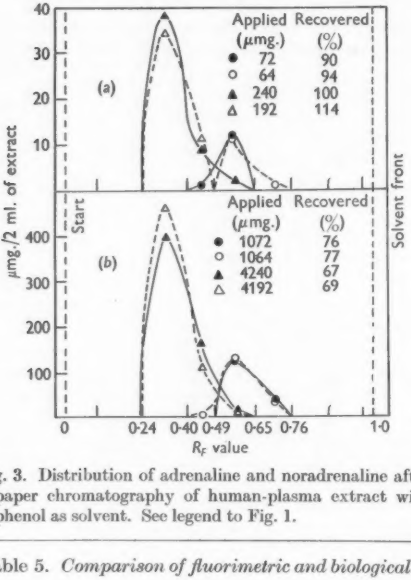

Fig. 3. Distribution of adrenaline and noradrenaline after paper chromatography of human-plasma extract with phenol as solvent. See legend to Fig. 1.

No evidence of hydroxytyramine was found. Its presence would have produced higher catecholamine estimates in the sample analysed by the ED method than in that analysed by the THI method; its position would have been in front of adrenaline in butanol chromatograms and just behind adrenaline in phenol chromatograms. The absence of significant amounts of hydroxytyramine, of course, had to be inferred from the similarity of the results obtained, even in crude extracts, with the ED and THI methods.

Bioassays

Each preparation of rat uterus and rat colon was tested with plasma extract, with pure standards and with a mixture of both (Table 5). Addition of plasma extract often decreased the response to the standard solution. A correction for this interference was made by graphical extrapolation; in these cases the figures for both the uncorrected and the corrected response are given. Since it may be assumed that the true response is greater than the uncor-

Table 5. *Comparison of fluorimetric and biological assays for adrenaline and noradrenaline in plasma extracts*

A, Adrenaline; N, noradrenaline.

Expt. no.	Source of blood	Concn. in plasma before purification (fluorimetric) A (μg./l. of plasma)	Concn. in plasma before purification (fluorimetric) N (μg./l. of plasma)	Final concentrate: Fluorimetric A (μmg./0·1 ml.)	Final concentrate: Fluorimetric N (μmg./0·1 ml.)	Final concentrate: Biological Uterus (as μmg. of A/0·1 ml.)	Final concentrate: Biological Colon (as μmg. of N/0·1 ml.)	Interference Uterus	Interference Colon
1	Ox	4·35	9·30	0·5	20	<0·5	<100	.	+ +
2	Ox	2·50	10·9	20	30	>0·3 <1·3	>20 <200	+	+
3	Ox	3·53	8·40	5	25	>1·4 <3·4	12·5	+	.
4	Ox	6·25	20·7	30	88	49	>44·5 <160	.	+
5	Ox	2·44	7·13	9	30	<0·3	<50	.	+ +
6	Ox	12·6	26·5	13	29	1·0	~10	.	.
7	Ox	2·92	6·0	6	13	<0·5	<50	.	.
8	Man	1·40	4·20	6	24	>0·07 <0·4	<50	+	+
9	Man	1·08	4·23	5	43	0·5	~5	.	.
10	Man	0·95	4·74	4	10·5	0·35	<50	.	.
11	Man	1·33	3·65	24	52	<0·5	<100	.	.
12	Man	—	—	8·5*	25*	<1·0*	<100*	.	.

* Fraction separated by paper chromatography with phenol solvent.

rected response and not higher than the corrected response, the figures for the uncorrected response are preceded by >, those for the corrected response by <. Very little interference was observed in the later experiments, probably because of a more thorough removal of salts.

The test for noradrenaline with rat colon is much less sensitive than the test for adrenaline with rat uterus, and the concentrations of noradrenaline indicated fluorimetrically were on the borderline of sensitivity. No definite conclusions can therefore be drawn from the results of this test. On the other hand, there was, according to the fluorimetric analysis, sufficient adrenaline to be detected without difficulty, yet satisfactory agreement was found only in Expt. 4 (Table 5); in Expt. 3 the bioassay was about 50 % of the fluorimetric assay. In all other experiments, whether with bovine or human blood, the biological activity was either nil or corresponded at the most to 10 % of the fluorimetric value.

Fluorimetric estimation indicated a fairly high adrenaline and noradrenaline concentration in the plasma of Expt. 4, and it may be argued that the relatively high biological activity in this case was due to a discharge of adrenal amines at the time of blood collection; however, very little biological activity was found in Expt. 6, where even higher concentrations were found fluorimetrically, and also in other samples of ox blood where the fluorimetric adrenaline estimate was much above the level found in the blood of resting human beings and where therefore a similar discharge might have been postulated.

DISCUSSION

It has sometimes been assumed that all catechol compounds form fluorescent derivatives with ED and that the THI method is specific for compounds capable of indole-ring closure and, in addition, possessing a β-hydroxyl group in the side chain. Both assumptions are oversimplifications. It has already been mentioned that, in terms of adrenaline fluorescence, dopa gives higher readings in the THI than in the ED method. Another such compound is adrenalone: its fluorescence in the ED method corresponds to about 0·6 % of that of an equivalent amount of adrenaline (read with yellow filter), whereas in the THI method the proportion is 4 %. The presence of dopa in plasma has been excluded. The same may be said of adrenalone; it would have been recognized, among other things, by its positions on paper chromatograms (R_F 0·50 in butanol–acetic acid–water, 0·65 in phenol–water). Interference by adrenochrome can also be ruled out, because this compound is not adsorbed by alumina at pH 8·4. With the exception of their higher *N*-alkyl homologues, adrenaline and noradrenaline are the only other substances known to form derivatives with comparable fluorescence intensities in both the ED and THI methods. It can hardly be assumed that the agreement between these two methods in the present experiments was fortuitous. It was found consistently, with both kinds of blood and at every stage of purification; it confirms earlier results (Bone, 1953; Weil-Malherbe & Bone, 1954*b*; Weil-Malherbe, 1955*b*) when the method of Lund (1950) was compared with the ED method. We are unable to account for the divergent results of Valk & Price (1956).

If therefore the agreement between the two fluorimetric methods is in itself a strong argument for the supposition that the substances estimated in plasma are actually adrenaline and noradrenaline, other results give it even stronger support. The cation-exchange experiments have shown that the basic fraction virtually accounts for all catechols in plasma, and that therefore acid, neutral or amphoteric components are absent. The fraction also has the same electrophoretic mobility as catecholamines. The chromatographic experiments have shown that hydroxytyramine is absent and that the substances estimated as adrenaline and noradrenaline move to their expected positions in two different solvent systems. The fact that these results were obtained by two independent methods seems to us to be of particular significance and to amount to virtual proof of the chemical identity of the two fractions with adrenaline and noradrenaline.

What then is the explanation for the failure to demonstrate a commensurate biological activity in the test with rat uterus? The fact that 60–70 % of plasma catecholamines are bound to platelets (Weil-Malherbe & Bone, 1954*b*) may account for the absence of biological activity in circulating blood or in platelet-rich plasma. It may, incidentally, also account for a low level of plasma catecholamines found by some investigators with the ED method; these, presumably, used more intensive centrifuging for the separation of plasma. It cannot, however, account for the absence of biological activity in purified extracts since a platelet suspension is stripped of bound catecholamines when it is passed through a column of alumina.

If racemization had occurred, e.g. through a catalytic effect of cation-exchange resins, biological activity should have corresponded to at least 50 % of the fluorimetric assay, but it was often 10 % or less.

In most biological tests normal responses were obtained with internal standards; there was therefore no obvious interference or technical flaw. It may be mentioned, however, that we also compared biological activity and fluorimetric assay in extracts of urine (Weil-Malherbe & Bone, 1957). While a satisfactory correlation was found in tests with rat colon, biological activity found in the uterus test exceeded 30 % of the fluorescence value for

adrenaline in only four out of 18 experiments. Euler & Floding (1955*b*), who compared the same fluorimetric method with biological activity by the test with chicken rectum, found that the biological activity exceeded 30% of the fluorimetric value in 13 out of 17 extracts of normal urine. The less-satisfactory agreement in our experiments suggests that there may be, after all, a technical reason for our results, but its explanation remains to be found.

One other improbable explanation is that the substance in plasma is the optical isomer of L-adrenaline. Imaizumi and co-workers (Imaizumi & Kawamoto, 1952; Imaizumi, Kawamoto, Kita & Sato, 1952; Imaizumi *et al.* 1954) described an enzyme in rabbit plasma which reversibly oxidizes L-adrenaline to adrenalone in the presence of diphosphopyridine nucleotide, and which they called adrenaline dehydrogenase. Such an enzyme may be expected to possess stereospecificity and might be useful for investigating the steric configuration of the substances present in plasma. So far we have not succeeded in confirming the claims of the Japanese authors. We have searched for this enzyme in rabbit and human plasma, and in extracts of rat liver, heart, spleen and brain. The experiments were done anaerobically to prevent any activity of amine oxidase; adrenaline was incubated with a large excess of diphosphopyridine nucleotide, but no disappearance was found by fluorimetric measurement. These experiments are being continued.

SUMMARY

1. Catechol compounds were extracted from bovine and human plasma by passage through columns of alumina and elution with dilute acid. The eluates were separated into basic and non-basic fractions by passage through cation-exchange resins. The purified basic fraction was studied by paper chromatography, paper electrophoresis or bioassay. At every stage of the procedure samples were analysed by two fluorimetric methods.

2. The basic fraction accounted for the catechol compounds present in the alumina eluates. Acid catechols, neutral catechols and 3:4-dihydroxyphenylalanine therefore do not occur in plasma. The estimates of adrenaline and noradrenaline obtained by direct fluorimetric assay agreed with the amounts recovered from the expected positions on paper after electrophoresis and after chromatography in two different solvent systems. There was no evidence for the presence of 3-hydroxytyramine.

3. If adrenaline and noradrenaline are the only catechol compounds in plasma the two fluorimetric methods used should yield identical results. A high degree of correlation was indeed found consistently at every stage of the procedure, including the paper chromatographic separation of fractions.

4. Bioassays of noradrenaline with rat ascending colon were inconclusive since the concentrations, which according to the fluorimetric analysis were available, were on the borderline of the sensitivity of the test. Little biological activity was found in tests for adrenaline with rat uterus in spite of chemical evidence for the presence of adrenaline.

5. Possible reasons for this discrepancy are discussed.

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Physiological Studies on Acid Metabolism

4. PHOSPHOENOLPYRUVIC CARBOXYLASE ACTIVITY IN EXTRACTS OF CRASSULACEAN PLANTS*

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A large increase in titratable acidity at night, and a concomitant decrease in carbohydrate, has long been recognized as a peculiar feature of the green tissues of Crassulacean and some other succulent plants (Wolf, 1939; Bennet-Clark, 1949). The increase in acidity in the dark is a result of the accumulation of malic acid, which in leaves (in air) may be as much as 800 mg./100 g. of fresh tissue during a single night (Vickery, 1953). It has also been shown, in earlier papers in this series (Thomas 1949; Thomas & Beevers, 1949; Thomas & Ranson, 1954; Ranson & Brown, in preparation) that this increase in acidity is accompanied by the fixation of respiratory carbon dioxide and may be augmented by supplying additional carbon dioxide. In the light, respiratory carbon dioxide is preferentially utilized in photosynthesis, but an increase in acidity in the light may be induced by the addition of carbon dioxide in excess of the photosynthetic requirement. It is clear then that the green tissues of Crassulacean plants possess unusually strong powers of carboxylation and provide suitable material for the study of the enzymes concerned in this process. Earlier investigations were concerned principally with malic and isocitric enzymes, and these were extended to include phosphoenolpyruvic carboxylase after its discovery in extracts of spinach leaves (Bandurski & Greiner, 1953). This, and an essentially similar enzyme in wheat germ (Tchen & Vennesland, 1955; Tchen, Loewus & Vennesland, 1955) catalyses the reaction

$$\text{Phosphoenolpyruvate} + CO_2 \xrightarrow{Mg^{2+}} \text{oxaloacetate} + \text{phosphate}. \quad (1)$$

Phosphoenolpyruvic carboxylase has been suggested (Tchen & Vennesland, 1955; Bandurski, 1955) to be an important enzyme in dicarboxylic acid accumulation in plant tissues, particularly in the Crassulaceae. As reported briefly elsewhere (Walker, 1956), this enzyme has now been obtained in crude extracts from Crassulacean plants and shown to function synthetically *in vitro*. Since this paper was prepared independent evidence of phosphoenolpyruvic carboxylase activity in homogenates of a Crassulacean plant has been reported by Saltman, Kunitake, Spolter & Stitts (1956). Details of the extraction and of the characteristics of the enzyme are given in this paper, and some aspects of the reaction are discussed in relation to the formation of acids in the dark.

* Part 3: Thomas & Ranson (1954).

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MATERIALS AND METHODS

The barium–silver salt of phosphoenolpyruvic acid, adenosine diphosphate and triphosphate (ADP, ATP), inosine diphosphate and triphosphate (IDP, ITP), guanosine diphosphate and triphosphate (GDP, GTP) and reduced diphosphopyridine nucleotide (DPNH) were obtained from the Sigma Chemical Corporation, St Louis, Missouri. *Kalanchoë crenata* and *Sedum spectabile* were grown by Mr M. Matheson in the garden of the Botany Department at Newcastle.

Preparation of enzyme extracts. Leaves were cut in the afternoon when their titratable-acid content was known to be at a minimum, and chilled at $-5°$ for 30–60 min. Meanwhile a weighed sample of the leaves was shredded into boiling water, which was then cooled and titrated against standard alkali. The titratable-acid value (ml. of 0·1 N-alkali/100 g. of leaf) thus derived was used to calculate the weight of 2-amino-2-hydroxymethylpropane-1:3-diol (tris) necessary to bring 100 g. of macerated leaf to pH 7·4. This quantity of tris was dissolved in 100 ml. of 1% (w/v) NaCl and the ice-cold solution placed in a chilled Waring Blendor. Chilled leaf (100 g.) was then finely and rapidly (within 10 sec.) shredded with a razor, quickly added to the Blendor and homogenized for 1 min. The homogenate was strained through muslin and centrifuged at 20000 *g* for 10 min. All the preparative manipulations were carried out in a cold room at $-5°$ within a period of 15 min.

Partial purification of extracts. The protein in the supernatant was precipitated by the addition of $(NH_4)_2SO_4$, at pH 7, to 60% saturation and the precipitate redissolved in, and dialysed against, 0·2% tris at pH 7·4. Alternatively, if the protein was resuspended in saturated $(NH_4)_2SO_4$ and stored at $-30°$ it was found to keep indefinitely without loss of activity. It could then be centrifuged down and redissolved as before.

Assay. Except where stated, reactions were followed with a Unicam SP. 500 spectrophotometer by measuring the change in optical density at 340 mμ associated with the oxidation of DPNH. Reactions were carried out in cells with a light path of 1 cm. in a temperature-controlled room at 25°. Each reaction mixture was made up to a final volume of 3 ml.

Chromatography. Standard methods (see Ranson, 1955) were used as a basis for all chromatographic analysis.

(*a*) Carboxylic acids: reactions were stopped by the addition of 1 drop of conc. HCl. Each mixture was tipped into a Soxhlet cup and dried in a vacuum desiccator. After ether extraction the acids were chromatographed on Whatman no. 1 paper with *tert.*-amyl alcohol–water–formic acid (3:3:1, by vol.) as the developing solution.

(*b*) Keto acids: reactions were stopped by the addition of 2:4-dinitrophenylhydrazine in 5 N-H_2SO_4. After incubation for 1 hr. the precipitated protein was centrifuged down and the supernatant extracted with ethyl acetate. The ethyl acetate solution was in turn extracted with 2% (w/v) Na_2CO_3 and then acidified and re-extracted with ethyl acetate. The final extract was evaporated to dryness *in vacuo* and redissolved in ethanol for chromatography with *tert.*-amyl alcohol–ethanol–water (50:10:40, by vol.) on Whatman no. 3 paper buffered at pH 6·2 with 0·2 M-phosphate buffer. Spots were eluted in 0·2 M-$NaHCO_3$ for determination of their ultraviolet-light-absorption spectra.

Determination of inorganic phosphate (after Allen, 1940). Reactions were stopped and the protein was precipitated by the addition of 0·4 ml. of 60% (w/w) $HClO_4$ to 2·2 ml. of the reaction mixture. The precipitate was removed by centrifuging at 2000 *g* for 1 min. To the supernatant were added 0·2 ml. of 10% (w/v) $Na_2S_2O_6$, 1% (w/v) amidol (2:4-diaminophenol hydrochloride) followed by 0·2 ml. of 8·3% (w/v) $(NH_4)_6Mo_7O_{24},4H_2O$. After 5 min. the intensity of the blue colour which developed was determined by readings at 660 mμ. The quantity of phosphate present was then obtained by reference to a standard curve.

Degradation of malic acid. The decarboxylation of malic acid was effected by a suspension of *Lactobacillus arabinosus* (Nossal, 1951). The carbon dioxide evolved was trapped in NaOH, from which it was precipitated as $BaCO_3$ by the addition of $BaCl_2$.

RESULTS

Production of oxaloacetate

After reaction (1) had been allowed to proceed for 15 min. in 3 ml. of a solution containing $MgCl_2$ (3 μmoles), phosphoenolpyruvate (10 μmoles), *Kalanchoë crenata* enzyme and tris buffer (0·3% at pH 7·4), it was stopped and the mixture tested for the presence of oxaloacetate (see Methods). Chromatography showed two well-defined spots which corresponded in position to those of the two 2:4-dinitrophenylhydrazones of oxaloacetic acid. After elution in Na_2CO_3, the absorption spectra of the known and suspected oxaloacetic hydrazones were determined and shown to be identical. The chromatograms also showed two faint spots with the R_F values of pyruvic hydrazone (see below).

When 1·6 mg. (50 μc) of $NaH^{14}CO_3$ was included in the reaction mixture and chromatograms were produced as before, the oxaloacetic hydrazones were found to be highly radioactive, but no activity was detected in the pyruvic spots. Since the decarboxylation of oxaloacetate, catalysed by Mg^{2+} ions, is known to occur at a rapid rate (Speck, 1949) it is inevitable that some, if not all, of this pyruvate might arise by decarboxylation of the oxaloacetate synthesized. The complete absence of labelling in the pyruvate therefore provided a very strong indication that the labelling in the oxaloacetate was restricted to the β-carboxyl group.

Production of inorganic phosphate in reaction (1)

Reaction mixtures were prepared as indicated in the legend to Fig. 1 and the reactions stopped at the times shown and their phosphate content determined. The results are given in Fig. 1. Production of phosphate was rapid at first and then decreased gradually as the reaction neared completion. No increase in inorganic phosphate could be detected in blanks, which were incubated with enzyme denatured by heat.

The linked reaction

All the extracts of Crassulacean plants were found to contain an active malic dehydrogenase which catalysed the reaction

$$\text{Oxaloacetate} + \text{DPNH} \rightarrow \text{malate} + \text{DPN}. \quad (2)$$

For purposes of measurement therefore the carboxylase and dehydrogenase reactions were linked, giving

$$\text{Phosphoenolpyruvate} + CO_2 + \text{DPNH} \xrightarrow{Mg^{2+}} \text{malate} + \text{phosphate} + \text{DPN} \quad (3)$$

and the oxidation of DPNH was followed spectrophotometrically. When measured independently the rates of inorganic phosphate accumulation in reaction (1), and DPNH oxidation in reaction (3), were found to be virtually identical.

The spectrophotometric demonstration of carboxylase activity in extracts of *Kalanchoë crenata* and *Sedum spectabile* is illustrated in Figs. 2 and 3. Partial purification of these crude preparations by $(NH_4)_2SO_4$ precipitation (see Methods) was sufficient

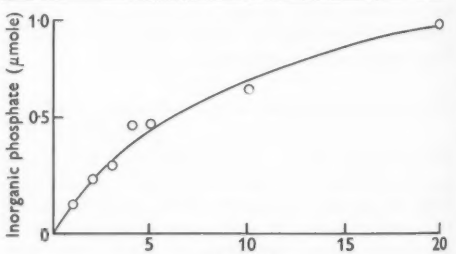

Fig. 1. Appearance of inorganic phosphate during the course of reaction (1). Each reaction mixture (3 ml.) contained *Kalanchoë* enzyme preparation, 1% (w/v) tris buffer at pH 7·4, $MgCl_2$ (3 μmoles) and potassium phosphoenolpyruvate (2 μ moles). Each reaction was stopped at the time indicated and the phosphate content determined.

to prevent the slight oxidation of DPNH in the absence of added phosphoenolpyruvate seen in Fig. 2. The requirement for added magnesium (Figs. 2, 3) could also be more clearly demonstrated after such treatment (see Table 2).

Typically, 100 g. fresh wt. of *Kalanchoë* leaf yielded 25 ml. of partly purified extract, in which the respective activities ($V_{max.}$) of malic dehydrogenase and phosphoenolpyruvic carboxylase were of the order of 10 and 0·5 μmoles of substrate utilized/min./ml. respectively.

Substrate and coenzyme requirement. Phosphoenolpyruvic carboxylase had been found (Tchen & Vennesland, 1955) in extracts of wheat germ to be associated with an enzyme which catalyses the reaction: phosphoenolpyruvate + CO_2 + ADP or IDP $\rightleftharpoons$ oxaloacetate + ATP or ITP. This 'phosphorylating decarboxylase' seems essentially similar to one in avian liver (Utter, Kurahashi & Rose, 1954; Utter & Kurahashi, 1954) for which GDP and GTP are even more effective than the corresponding inosine nucleotides (Kurahashi & Utter, 1955). The reaction is reversible but it also appears to differ from that catalysed by phosphoenolpyruvic carboxylase in that its principal role is probably in oxidative phosphorylation rather than carboxylation (Utter & Kurahashi, 1954; Tchen & Vennesland, 1955; Bartley, 1955). Added nucleotides were without effect on the *Kalanchoë* enzyme (Table 1) and it has already been seen that inorganic phosphate was produced during the course of the reaction (Fig. 1).

No reaction occurred if potassium pyruvate (either alone or together with ATP, or ITP or GTP, in tris or phosphate buffer at pH 7) was substituted for phosphoenolpyruvate.

Cofactor requirement. The rate of oxidation of DPNH when manganese and other cations were substituted for magnesium in the reaction mixture is shown in Table 2. Manganese is seen to have been as effective as magnesium, although other results

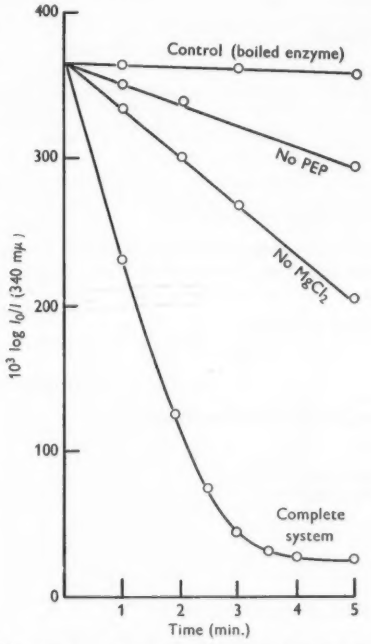

Fig. 2. Reaction (3) catalysed by *Kalanchoë* extracts. Each reaction mixture (3 ml.) contained *Kalanchoë* enzyme preparation, 1% (w/v) tris buffer at pH 7·4, DPNH (approx. 0·2 μmole), sodium phosphoenolpyruvate (1 μmole), $MgCl_2$ (3 μmoles), except for the omissions indicated. The reaction was started by the addition of phosphoenolpyruvate (PEP) and DPNH, except where the former was omitted. The curves have been equated to the same starting point.

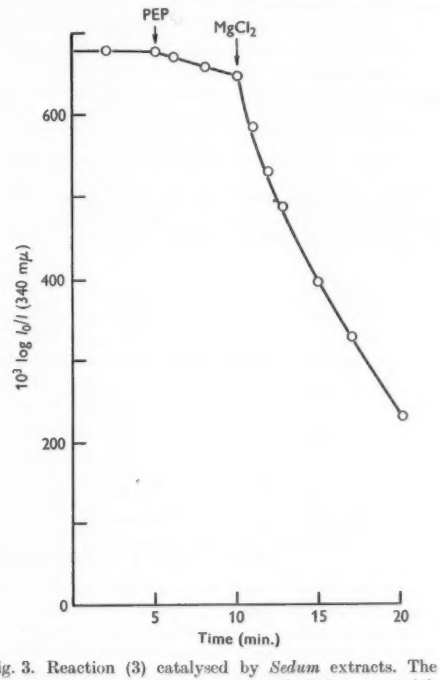

Fig. 3. Reaction (3) catalysed by *Sedum* extracts. The reaction mixture (3 ml.) contained *Sedum spectabile* enzyme preparation, 1% (w/v) tris buffer at pH 7·4, DPNH (approx. 0·3 μmole) added at zero time, sodium phosphoenolypyruvate (PEP; 1 μmole) and $MgCl_2$ (3 μmoles) added as indicated.

have indicated that this may not be the case after prolonged dialysis. Except for cobalt and cadmium the other cations were not only ineffective but even inhibitory when used in addition to magnesium (see Table 2).

Effect of pH. In phosphate buffer the linked reaction (3) was most rapid at about pH 7. At either side of this pH value the reaction is less rapid, falling to about 75 % of the maximum at pH 6 and 8. The rate of the dehydrogenase reaction alone, acting in the direction of malate synthesis, falls steadily over this range as the pH rises, which suggests that the optimum of the carboxylase is, if anything, above pH 7.

Effect of inorganic phosphate. Tchen & Vennesland (1955) found that low concentrations of inorganic phosphate accelerated the reaction. Table 3 illustrates a similar effect with the *Kalanchoë* enzyme. It will be seen, however, that above an optimum concentration further increases in phosphate concentration brought about a progressive inhibition.

Substrate concentration. Increases in either the concentration of phosphoenolpyruvate or carbon dioxide brought about corresponding increases in the reaction rate. Laidler & Socquet (1950) have derived an equation for the initial velocity of a two-substrate enzyme system in which each type of substrate molecule occupies its own site on the enzyme surface. For relatively small concentrations

Table 1. *Effect of several coenzymes on the rate of reaction* (3)

Each spectrophotometer cell contained enzyme, $MgCl_2$ (3 μmoles), DPNH (0·2 μmole), tris buffer (1 %, w/v) at pH 7·4, and sodium phosphoenolpyruvate (2 μmoles) together with the additions indicated, in a volume of 3 ml. The rate given is the change in log I_0/I (340 mμ) × 1000 in the first 60 sec. of the reaction.

		Additions						
	Control (no additions)	Cocarboxylase (2 μmoles)	IDP (2 μmoles)	ITP (2 μmoles)	GDP (2 μmoles)	GTP (2 μmoles)	Inorganic phosphate (2·5 μmoles)	IDP + inorganic phosphate (2·5 μmoles)
Rate	88	85	87	88	86	84	97	93

Table 2. *Effect of several cations on the rate of reaction* (3)

Each spectrophotometer cell contained enzyme, DPNH (0·2 μmole), tris buffer (1 %, w/v) at pH 7·4, and sodium phosphoenolpyruvate (2 μmoles) together with the additions indicated, in a final volume of 3 ml. The rate given is the change in log I_0/I (340 mμ) × 1000 in the first 60 sec. of the reaction.

		Added cations (3 μmoles)								
	Control (no additions)	Mg^{2+}	Mn^{2+}	Cd^{2+}	Co^{2+}	Ca^{2+}	Ni^{2+}	Zn^{2+}	Cu^{2+}	Fe^{2+}
Rate	7	90	100	31	28	5	4	2	Precipitation	

Inhibition (%) by cations (3 μmoles) in the presence of magnesium (3 μmoles)

	%
Fe^{2+}	100 precipitating protein
Cu^{2+}	100 precipitating protein
Zn^{2+}	98
Ni^{2+}	82
Ca^{2+}	30

Table 3. *Effect of orthophosphate concentration on the rate of reaction* (3)

Each spectrophotometer cell contained enzyme, sodium phosphoenolpyruvate (2 μmoles), $MgCl_2$ (3 μmoles), DPNH (0·2 μmole) and tris buffer (1 %, w/v) at pH 7·4, and the quantities of orthophosphate (also at pH 7·4) indicated, in a final volume of 3 ml. The rate given is the change in log I_0/I (340 mμ) × 1000 in the first 60 sec. of the reaction. In the absence of added phosphate the reaction mixture contained less than 0·05 μmole of free inorganic phosphate, and the observed change in optical density due to the reaction corresponds to an increase of about 0·04 μmole.

	Phosphate added (μmoles)							
	0	1	2	3	15	30	150	300
Rate	112	120	128	152	144	116	68	36

their equation may be simplified and rewritten (Schwert & Hakala, 1952) as

$$\frac{1}{V}=\frac{K_a}{V_{max.}}\left[\frac{K_b}{[b]}+1\right]\frac{1}{[a]}+\frac{1}{V_{max.}}\left[\frac{K_b}{[b]}+1\right],$$

where a and b are the two substrates and K_a and K_b the respective dissociation constants. In this form the expression is linear for $1/V$ against $1/[a]$ at constant [b]. Alternatively, written

$$\frac{1}{V}=\frac{K_b}{V_{max}}\left[\frac{K_a}{[a]}+1\right]\frac{1}{[b]}+\frac{1}{V_{max.}}\left[\frac{K_a}{[a]}+1\right],$$

the expression is linear for $1/V$ against $1/[b]$ at constant [a].

On this basis the kinetic constant (K_a) for phosphoenolpyruvate was evaluated by plotting the reciprocals of velocity against the reciprocals of phosphoenolpyruvate concentration at constant carbon dioxide concentration.

The straight line so obtained was then extrapolated (cf. Dixon, 1953) to provide the value of $-1/K_a$ at its intersection with the x axis (Fig. 4). The value of K_a derived in this way was $1{\cdot}94\times10^{-4}$ mole/l., and a value of $1{\cdot}5\times10^{-4}$ was similarly obtained from measurements of inorganic phosphate accumulation in reaction 1. The evaluation of reaction rates by inorganic phosphate determinations was rather less accurate than by the spectrophotometric method, and the discrepancy between the two results is not considered to be greater than experimental error. The fact that the rates of reactions 1 and 3 clearly corresponded closely over a wide range of concentrations justifies the use of the spectrophotometric method of obtaining kinetic constants.

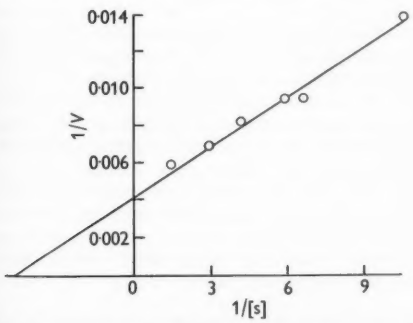

Fig. 4. Increase in the rate of reaction (3) with increasing phosphoenolpyruvate concentration. Each spectrophotometer cell contained *Kalanchoë* enzyme preparation, 1% (w/v) tris buffer at pH 7·4, DPNH (0·2 μmole), $MgCl_2$ (3 μmoles) and sodium phosphoenolpyruvate in the concentrations (μmoles/ml.) indicated by the reciprocal, in a final volume of 3 ml.

The value of K_a is of the same order as that of 5×10^{-4} obtained by Bandurski (1955) under slightly different conditions. Similar results were obtained when the carbon dioxide concentration was varied at constant phosphoenolpyruvate concentration (Walker & Brown, 1957); the value of the dissociation constant (K_b) was found in this case to be $2{\cdot}2\times10^{-4}$ mole/l.

Enzyme concentration. Within the limits of measurement on the spectrophotometer, the rate of reaction was directly proportional to the enzyme concentration, being doubled as the enzyme concentration was doubled.

Synthesis of malate in the linked reaction

The fixation of carbon dioxide in oxaloacetate in reaction mixtures containing the *Kalanchoë* enzyme preparation has been described at the beginning of this section. If DPNH also was added to these solutions the oxaloacetate was reduced to malate (reaction 3) and the concomitant oxidation of DPNH could be followed in the spectrophotometer. Chromatographic analysis of such reaction mixtures gave spots which had the same R_F values as malic acid chromatographed on the same sheets. When 50 μc of radioactive bicarbonate was also added to the reactants the malate synthesized became highly active. After co-chromatography with malate the identity of the synthesized acid was then confirmed by radioautographs, which showed the major part of the activity to emanate from the malate spots. Some activity (less than 1% of the total) was found to be associated with the fumaric spots, probably as a result of a weak fumarase activity in the enzyme preparation. When active malate was eluted from the chromatograms and degraded by *Lactobacillus* (see Methods) 99·7% of the counts were recovered in carbon dioxide originating from the β-carboxyl group. The residue, which would contain all the activity associated with the other carbon atoms, together with any activity not released by the *Lactobacillus*, contained only 0·3% of the total counts.

DISCUSSION

If it is accepted that the synthesis of malate which occurs in Crassulacean plants during the night is brought about at the expense of carbohydrate breakdown, it may be assumed that phosphoenolpyruvate is an intermediate since this substance is produced directly by the Embden–Meyerhof–Parnas pathway and also indirectly by the pentose phosphate carboxylating cycle. If carboxylation occurs after the formation of phosphoenolpyruvate the most likely system would involve either malic enzyme or phosphoenolpyruvic carboxylase.

If the former system operates the sequence would involve reactions (4) and (5):

$$\text{Phosphoenolpyruvate} + \text{ADP} \rightarrow \text{pyruvate} + \text{ATP}, \quad (4)$$

$$\text{Pyruvate} + CO_2 + \text{TPNH} \xrightarrow{Mn^{2+}} \text{malate} + \text{TPN}. \quad (5)$$

(TPN, triphosphopyridine nucleotide; TPNH, reduced triphosphopyridine nucleotide.) Malic enzyme, which catalyses reaction (5), has frequently been suggested as an important agent in carboxylation. The equilibrium position is unfavourable, but the reaction may be driven in the direction of reductive carboxylation if it is linked with a system which generates reduced TPN (Ochoa, 1951). Harary, Korey & Ochoa (1953) have shown that at pH 7 and at 22° in an atmosphere containing 5% of carbon dioxide and equimolar concentrations of oxidized and reduced TPN, the ratio pyruvate:malate would be approx. 1:0·035. The actual ratio in the *Kalanchoë* leaf, on the other hand, is probably never less than 1:100 (Bradbeer, 1954). It seems reasonable to assume therefore that in any explanation of acidification (in *Kalanchoë*) based upon the malic enzyme, a place must be found, not only for the regeneration of reduced TPN but also for some other system by which malate is removed from the site of synthesis at an extremely rapid rate.

The alternative sequence would involve reactions (1) and (2). According to Tchen & Vennesland (1955), the equilibrium position of reaction (1) is such that for all practical purposes the reaction is irreversible. From the value of the equilibrium constant of reaction (2) (Stern, Ochoa & Lynen, 1952) it may be calculated that, at pH 7·4 in the presence of equimolar concentrations of oxidized and reduced DPN, the ratio oxaloacetate:malate at equilibrium would be about 1:43 000. Moreover, the value of the equilibrium constant is strongly dependent on pH, and under the acid conditions in the leaf the actual ratio might be much higher. Acidification via this sequence could therefore occur at near maximal rates even if malate was not rapidly removed from the site of synthesis.

Whichever system operates the total free-energy changes are of the same order, but there are important differences in the way in which this energy is conserved. Thus it appears that in reaction (1) the large negative free energy associated with the hydrolysis could be directly utilized in the carboxylation. Results obtained by Bandurski (1955) indicate that the reversible formation of orthophosphate and a pyruvate–enzyme complex is unlikely. Moreover, though Tchen *et al.* (1955) do not rule out oxaloacetyl phosphate as a possible intermediate, they have clearly shown that the phosphoenol P–O bond of phosphoenolpyruvate must be broken before, or simultaneously with, the addition of carbon dioxide. J. G. Buchanan (private communication) has suggested that the latter possibility might be essentially similar to that postulated by Srinivasan, Katagiri & Sprinson (1955) for the synthesis of shikimic acid (see Fig. 5).

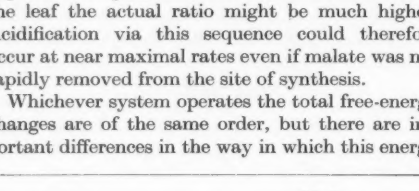

Fig. 5. Suggested mechanism of reaction (1).

If the malic enzyme system operates, the direct utilization of energy in this way would not be possible, although the ATP generated in reaction (4) might bring about the regeneration of reduced TPN required for reaction (5) (Krebs, 1954).

If both systems operate in the leaf it might be thought that competition would occur between reactions (1) and (4). Then the course of acidification would depend both on the amounts of the respective enzymes present and on their respective affinities for phosphoenolpyruvate. There would also be competition between reactions (1) and (5) for the available carbon dioxide. The importance of this will be discussed more fully in a second paper (Walker & Brown, 1957).

SUMMARY

1. The β-carboxylation of phosphoenolpyruvate to yield oxaloacetate and inorganic phosphate has been catalysed by cell-free extracts of Crassulacean plants.

2. The phosphoenolpyruvic carboxylase and malic dehydrogenase in these extracts have been linked to bring about malate synthesis.

3. The conditions under which the extracts bring about the catalysis of the linked reaction have been studied and the effect of substrate concentration has been determined.

4. Some aspects of the linked reaction and of the other reactions which bring about the conversion of phosphoenolpyruvate into malate have been considered in relation to the increase in acidity in the dark in Crassulacean plants.

The author wishes to express his indebtedness to Professor M. Thomas, F.R.S., at whose suggestion this work was undertaken, and to thank Miss J. Moyle, Dr R. Hill, F.R.S., and Dr S. L. Ranson for their invaluable advice and criticism. He is also grateful to Miss M. Stiller and Dr J. G. Buchanan for their comments on specific points.

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Physiological Studies on Acid Metabolism

5. EFFECTS OF CARBON DIOXIDE CONCENTRATION ON PHOSPHOENOLPYRUVIC CARBOXYLASE ACTIVITY*

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(*Received* 15 *January* 1957)

The demonstration that the increase in acidity in leaves of Crassulacean plants in the dark is accompanied by the fixation of respiratory carbon dioxide and can be augmented by feeding with this gas (Thomas, 1947, 1949; Thomas & Beevers, 1949) made it highly probable that the concentration of carbon dioxide would affect the rate of acid synthesis. In parallel work, Bonner & Bonner (1948), though not actually demonstrating carbon dioxide fixation, observed a sharp increase in the rate of acid synthesis in *Bryophyllum* leaves on increasing the external carbon dioxide concentration from zero to 0·1 %, and a further slow rise to a maximum at higher concentrations.

Ranson's measurements of acidification and carbon dioxide fixation in *Kalanchoë* leaves reported by Thomas (1951) and by Thomas & Ranson (1954) covered a more extensive range of concentration, and disclosed that beyond 3 % further increases in carbon dioxide depressed the rate of acidification.

From the earlier work it could be inferred that one or other of the enzymes responsible for malate synthesis in *Kalanchoë* leaves should (*a*) be able to carboxylate a carbohydrate cleavage product; (*b*) have a high affinity for carbon dioxide. And, in addition, that one or more of the reactions involved should be (*c*) accelerated by augmenting carbon dioxide concentration at low levels; (*d*) inhibited by high concentrations of carbon dioxide.

In the earlier paper (Walker, 1957) phosphoenolpyruvic carboxylase extracted from *Kalanchoë* leaves has been shown to fulfil the first of these requirements. In the present paper the other requirements are also shown to be satisfied and these findings are contrasted with the known characteristics of malic enzyme. Some aspects of the observed carbon dioxide inhibition are also discussed.

So far as is known this is the first time that a direct inhibitory effect of high carbon dioxide concentrations on an enzyme has been observed *in vitro*, although Thomas (1925) virtually inferred that carbon dioxide can inhibit the aerobic enzymic consumption of a zymase cleavage product.

* Part 4. Walker (1957).

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MATERIALS AND METHODS

All solutions, other than those containing the enzyme, were prepared in boiled mineral-free water which had been allowed to cool in a stoppered vessel containing no air. Residual carbon dioxide which remained in the reaction mixtures despite these precautions was measured manometrically as follows. Immediately after each reaction (i.e. within 3 min. of the start) the contents of the spectrophotometer cell were tipped into a Warburg flask. After equilibration for 2 min., acid was added from the side arm and the volume of evolved carbon dioxide was recorded.

The concentration of added carbon dioxide was varied as required by the addition of carbon dioxide-saturated bicarbonate at pH 7·4. Quantities were calculated from the Henderson–Hasselbalch equation (Umbreit, Burris & Stauffer, 1949) to give solutions in equilibrium with atmospheres containing the percentage of carbon dioxide required. For low concentrations, the total of added and residual carbon dioxide was measured manometrically as above. Totals obtained in this way were found to be in excellent agreement with the calculated figures if the residual carbon dioxide was taken to be constant in each case.

Materials and other methods were as described in the earlier paper (Walker, 1957).

RESULTS

Both the carboxylation of phosphoenolpyruvate

$$\text{Phosphoenolpyruvate} + CO_2 \rightarrow \text{oxaloacetate} + \text{phosphate} \quad (1)$$

and the reduction of oxaloacetate

$$\text{Oxaloacetate} + \text{DPNH} \rightarrow \text{malate} + \text{DPN} \quad (2)$$

are catalysed by cell-free extracts of *Kalanchoë crenata* (Walker, 1957), and the two reactions may be linked to give the overall reaction

$$\text{Phosphoenolpyruvate} + CO_2 + \text{DPNH} \rightarrow \text{malate} + \text{phosphate} + \text{DPN}. \quad (3)$$

(DPN, diphosphopyridine nucleotide; DPNH, reduced diphosphopyridine nucleotide.) Reaction (3) was found to proceed at a rapid rate, even when precautions were taken to reduce to a minimum the carbon dioxide present in the reagents. The source of carbon dioxide was then believed to be a small residue which could not be readily removed from the enzyme preparation. Fig. 1 shows results of an experiment in which initial reaction rates were determined at several low carbon dioxide concentrations. Rate is plotted against the sum of residual and added carbon dioxide; this total was determined manometrically (see Methods).

In this experiment the residual carbon dioxide was found to be 18·3 μl. in 3 ml., and evidently the reaction was able to proceed initially at this concentration at almost two-thirds of the maximal rate.

It will be seen that the addition of further carbon dioxide brought about increases in the reaction rate but it appears that the system became saturated at a relatively low level. The curve is seen to permit a reasonable extrapolation to zero rate at zero carbon dioxide concentration. Plotted as the double

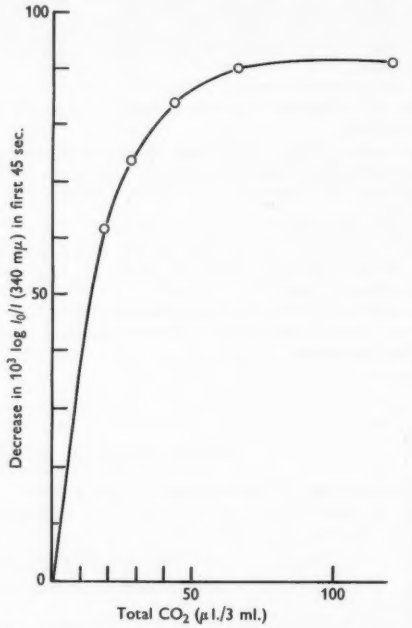

Fig. 1. Increases in the rate of reaction (3) brought about by small increases in the carbon dioxide concentration. Each spectrophotometer cell contained *Kalanchoë* enzyme preparation, 1% (w/v) tris buffer at pH 7·4, $MgCl_2$ (3 μmoles), sodium phosphoenolpyruvate (1 μmole), DPNH (0·2 μmole) and carbon dioxide-saturated bicarbonate at pH 7·4 to yield the carbon dioxide content indicated (see Methods), in a total volume of 3 ml.

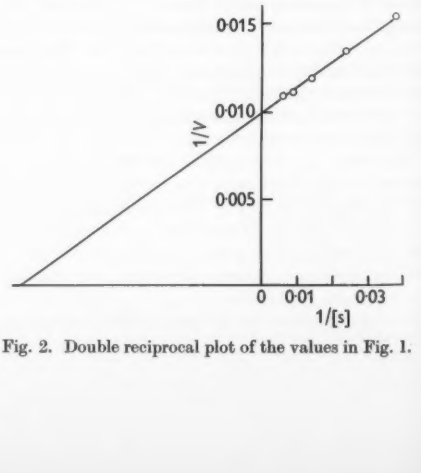

Fig. 2. Double reciprocal plot of the values in Fig. 1.

Table 1. *Effects of carbon dioxide concentration on phosphoenolpyruvic carboxylase activity and on the accumulation of malic acid in leaves in the dark*

For the spectrophotometric measurements (line 2) each cell contained *Kalanchoë* enzyme preparation, 1% (w/v) tris buffer at pH 7·4, $MgCl_2$ (3 μmoles), sodium phosphoenolpyruvate (1 μmole), DPNH (0·2 μmole) and carbon dioxide-saturated bicarbonate at pH 7·4 (see Methods), in a total volume of 3 ml. Reactions were started by the addition of phosphoenolpyruvate, and the figures refer to the decrease in log I_0/I (340 mμ) × 1000 in the period between 15 and 60 sec. after this addition. All figures in the third line (taken from Thomas & Ranson, 1954) are mean values for a number of experiments, except those which are underlined. These latter were derived from single experiments only, and the value 37 would appear to be an aberrant.

CO_2 (%)	0–0·05	0·05	0·125	0·25	0·5	1·0	2·0	3·0	5	10	15	30	50	70
Inorganic phosphate produced in 12 min. (see text) (μmoles)	0·59	—	—	—	1·55	1·2	—	—	1·15	1·03	—	0·43	0·30	0·2
Decrease in log I_0/I (340 mμ) in 45 sec. (see text)	64	74	84	90	91	84	—	79	—	66	—	36	18	11
Accumulation of malic acid in *Kalanchoë* leaf (mg./hr./100 g. fresh wt.)	19	22	—	—	—	60	62	67	45	24	16	37	4	1

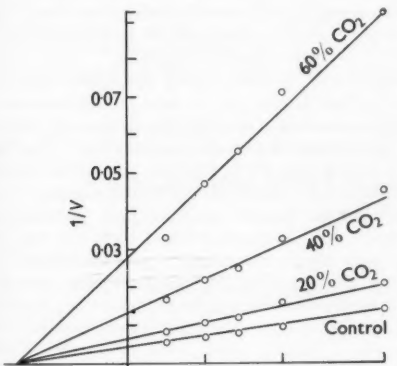

Fig. 3. 'Non-competitive' nature of carbon dioxide inhibition of reaction 3. Each spectrophotometer cell contained *Kalanchoë* enzyme preparation, 1% (w/v) tris buffer at pH 7·4, $MgCl_2$ (3 μmoles), DPNH (0·2 μmole) and sodium phosphoenolpyruvate (PEP) in the concentrations (μmoles/ml.) indicated by the reciprocal 1/[s]. Carbon dioxide concentration was adjusted as described (see Methods).

reciprocal (Lineweaver & Burk, 1934) these figures yield points which approximate to a straight line. This has been extrapolated to obtain a value of $-1/K_s$ at its intersection with the x axis (Fig. 2). The value of the constant derived in this way is $K_s = 2{\cdot}2 \times 10^{-4}$ mole/l. (also see Walker, 1957).

Increases in carbon dioxide concentration above the saturation level and to about 3% did not affect the reaction rate to any large extent, but further increases were progressively inhibitory (see Table 1, line 2).

In another experiment initial reaction rates were measured at several concentrations of phosphoenolpyruvate in the presence of each of three such inhibitory levels of carbon dioxide. Fig. 3 shows the results plotted as the double reciprocals (cf. Lineweaver & Burk, 1934). A family of straight lines is obtained and these lines coincide at a point on the x axis equivalent to $-1/K_s$. This is clearly indicative of a purely non-competitive type of inhibition as defined by Friedenwald & Maengwyn-Davies (1954).

A number of control experiments were carried out which showed that carbon dioxide (at several concentrations between 0·05 and 90%) did not alter the rate of the reaction catalysed by malic dehydrogenase (reaction 2). That it was, in fact, the phosphoenolpyruvic carboxylase reaction that was affected was confirmed by direct measurement. In the absence of reduced DPN this reaction does not proceed beyond the production of oxaloacetate and inorganic phosphate (reaction 1). Table 1 (line 1) shows the amount of phosphate produced when reaction mixtures containing enzyme, 2 μmoles of phosphoenolpyruvate, 3 μmoles of $MgCl_2$ and carbon dioxide as indicated were incubated in 2% (w/v) tris buffer at pH 7·4 for periods of 12 min. The response to changes in the carbon dioxide concentration is in the same direction as that observed when the oxidation of reduced DPN was followed in the linked reaction (Table 1, line 2). For the phosphate measurement the 'residual' carbon dioxide (which was present in the reaction mixture despite attempts to exclude it) was not evaluated but is expressed as 0–0·05%.

DISCUSSION

In a previous paper (Walker, 1957) two enzymic systems have been discussed which might bring about the conversion of phosphoenolpyruvate into malate during the increase in acidity in the dark in Crassulacean plants (see criterion (*a*) in the introduction). These involve reactions (1) and (2) or, alternatively, reactions (4) and (5):

$$\text{Phosphoenolpyruvate} + \text{ADP} \rightleftharpoons \text{pyruvate} + \text{ATP}, \quad (4)$$

$$\text{Pyruvate} + \text{CO}_2 + \text{TPNH} \rightleftharpoons \text{malate} + \text{TPN}. \quad (5)$$

(TPN, triphosphopyridine nucleotide; TPNH, reduced triphosphopyridine nucleotide.) One of the main criticisms of the malic enzyme reaction (5) as an important carboxylating system is its high carbon dioxide requirement. Thus from Ochoa's figures (Ochoa, 1951) it is seen that the highest rate of synthesis was achieved in the highest concentration (70 %) of carbon dioxide used, whereas in the presence of 5 % carbon dioxide this rate had fallen to about 27 % of the maximum. Assuming a linear decline in activity, we may infer that the rate of synthesis would have fallen to a very low level had this concentration been reduced still further. Yet rapid increase in acidity in the dark, at the expense of respiratory carbon dioxide, has been shown to occur (Ranson, reported by Thomas, 1951; Wood, 1952; Thomas & Ranson, 1954) in the leaves of Crassulacean plants maintained in atmospheres containing no carbon dioxide. In fact the affinity for respiratory carbon dioxide is so high that, for leaves enclosed in ordinary air, the value of the apparent respiratory quotient (carbon dioxide actually evolved/oxygen absorbed), as has long been known (Thomas, 1949; Wolf, 1938), is at first very low and may be zero. In one experiment on *Opuntia* the darkened shoot organs removed all the carbon dioxide from the surrounding air in addition to consuming respiratory carbon dioxide (Thomas & Ranson, 1954).

No doubt it can be very misleading to attach too much importance to the equilibrium constants of enzymes which are functioning in a dynamic system. Many overall reactions are known in which at least one step proceeds in a direction contrary to that which might have been expected from its equilibrium constant. In fact reaction (5) *in vitro* has often been driven in the direction for carboxylation by coupling with other reactions which regenerate reduced TPN and by reactions which tend to remove the malate which is synthesized. Nevertheless, *in vivo* the concentration of pyruvate available is probably never more than 5 mg./100 g. fresh wt. of leaf at any one time (Bradbeer, 1954), and it has been seen that rapid acidification can occur in leaves exposed to conditions which would not be expected to favour high levels of carbon dioxide. Since the equilibrium position of reaction (5) favours decarboxylation to a very large extent it seems improbable that this system, acting under these conditions, could bring about the large synthesis of malic acid which is known to occur. On this basis alone the facts point to a reaction which favours carboxylation and to an enzyme with a very great affinity for carbon dioxide. Phosphoenolpyruvic carboxylase fulfils both of these requirements. Both Tchen & Vennesland (1955) and Bandurski (1955) have commented on the high affinity for carbon dioxide exhibited by this enzyme (see introduction criterion (*b*)). Figures given in this paper indicate that reaction (1) *in vitro* functions at near maximal rates in the presence of very small quantities of carbon dioxide. This suggests not only that this enzyme system might bring about acidification at low carbon dioxide tensions, it implies also that *in vivo* this enzyme might offer strong competition to other carboxylating systems. Thus an active phosphoenolpyruvic carboxylase could preferentially utilize substrates of both reactions (4) and (5) of the alternative sequence.

Additional evidence that phosphoenolpyruvic carboxylase might play a role in the increase in acidity occurring in the dark is given by its behaviour in high carbon dioxide concentrations. It has been seen that below 0·5 % increases in carbon dioxide concentration caused concomitant increases in the rate of the linked reaction [see introduction, criterion (*c*)]. Between 0·5 and 10 % the rate falls slowly to about two-thirds of the maximum, and above 10 % further increases brought about a progressive inhibition [see introduction, criterion (*d*)]. In Table 1 (line 3) are given results obtained by Thomas & Ranson (1954), which show the effect of carbon dioxide on increase in acidity in the intact leaf in the dark. Comparison with the results *in vitro* (Table 1, lines 1 and 2) will show that in both instances there is an initial acceleration, a broad plateau and finally a depression or inhibition. Though in no way conclusive, this parallelism is certainly consistent with the view that phosphoenolpyruvic carboxylase is concerned in the increase in acidity in whole leaves in the dark.

There is little in the present data which can be used to define the actual nature of the observed inhibition by carbon dioxide. The kinetic picture clearly corresponds to that outlined by Friedenwald & Maengwyn-Davies (1954) for purely non-competitive inhibition. This casts doubt on the view that carbon dioxide could compete with phosphoenolpyruvate for the site at which the latter becomes attached to the enzyme surface. If each substrate molecule has its own point of attachment to the enzyme then steric interference with its own activa-

tion, by an excess of carbon dioxide acting at its own site, seems to us the most feasible of the possibilities which could produce the observed kinetic behaviour.

SUMMARY

1. Two different methods have been used to determine the effects of carbon dioxide on the rate of the reaction catalysed by phosphoenolpyruvic carboxylase.

2. The kinetics of the carbon dioxide effects have been studied with one of these methods in a reaction in which the carboxylase was linked with malic dehydrogenase.

3. The results have been discussed in relation to factors which are known to affect the rate of increase of acidity in the dark in Crassulacean plants.

The authors wish to express their indebtedness to Professor M. Thomas, F.R.S., at whose suggestion this work was undertaken, and to thank Miss J. Moyle, Dr R. Hill, F.R.S., and Dr S. L. Ranson for their invaluable advice and criticism. They are also grateful to Dr G. Maengwyn-Davies for her comments on specific points. Part of this work was carried out under grants from the D.S.I.R., and part during the tenure of an I.C.I. Fellowship held by one of us (D. A. W.).

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The Amino Acid Composition of Amphibian, Reptile and Avian Gelatins

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The present paper completes an investigation of the variation in amino acid composition found among the collagens and derived gelatins of the more important groups of vertebrates. Results are given for avian, reptilian and amphibian gelatins for comparison with the two previous sections of the work on mammalian (Eastoe, 1955) and fish (Eastoe, 1957) materials. Neuman (1949) included both chicken tendon and the subcutaneous membrane of the turtle in his survey, in which microbiological methods were used. No values for amphibian proteins of this type appear to have been published previously.

It has already been demonstrated (Eastoe, 1955) that, within the limits of experimental error, a carefully prepared gelatin adequately represents the composition of its collagen precursor, except in amide nitrogen. This is especially valuable when non-collagenous impurities, which cannot readily be removed, are present in a tissue and would introduce errors if the whole tissue were analysed. The materials selected for analysis were gelatins prepared from toad, python and crocodile skins and from chicken-leg tendon. In this way representative values for the gelatins and collagens of three further classes of vertebrates were obtained by ion-exchange chromatography (Moore & Stein, 1951), a technique identical with that of the previous papers being used.

Following the suggestion of Gustavson (1953) that shrinkage temperatures are a measure of structural stability, these have been measured for each of the collagens studied.

EXPERIMENTAL

Materials

Python-skin gelatin. Python (probably *Python sebae*). The skin was limed at 0° for 2 months, washed, and gelatin extracted from it by heating with water for 2 hr. at 60° and pH 7.

Crocodile-skin gelatin. Crocodile skin from the back was soaked in 5% (w/v) HCl for 4 days at room temp. and then extracted with water by heating for 2 hr. at 80° and pH 3. The air-dried gelatin obtained was dissolved in water and adjusted to pH 7. The gelatin was precipitated at 40° by adding a suitable quantity of ethanol at the same temperature. After 2 hr. the supernatant liquor could be decanted off. The precipitated gelatin was swollen in 10 ml. of water, dissolved by warming, allowed to set to a gel and dried.

Chicken-leg-tendon gelatin. Chicken (*Gallus*) leg tendons were soaked overnight in SO_2-saturated water, and washed several times with water. Further water was added and saturated with SO_2, and the tendons were extracted at 80° for 3 hr. The liquor was filtered and the bulk of the SO_2 removed with N_2. The solution was neutralized, allowed to set to a gel and dried. Approximately half the ash content could be accounted for in terms of Na_2S and Na_2SO_4 resulting from the ashing of the Na_2SO_3 formed during neutralization.

Toad-skin gelatin. Skin from the back, belly and limbs of the South African clawed toad *Xenopus laevis* was washed with ether and water to remove mucus, soaked in 10% (w/v) NaCl (three changes for 4 days each) and the outer keratinous layer pulled off and discarded. The collagenous layer was washed successively with water, acetone and ether and finally air-dried. The skins were soaked in 0·02 N-HCl for 24 hr., the pH being kept below 3, and then extracted for 3 hr. at 70° with the same liquor. The solution was adjusted to pH 7, filtered, and evaporated to dryness.

Methods

Hydrolysis. About 0·2 g. of a gelatin was hydrolysed with 20 ml. of 20% (w/w) HCl at 100° in a sealed tube for 24 hr.

Amino acid analysis. The analyses were carried out by the method of Moore & Stein (1951) as described by Eastoe (1955); the corrections applied were the same. Hydroxyproline was determined by the method of Neuman & Logan (1950).

Moisture. The loss of weight on drying a thin film of the gelatin at 105° for 24 hr. was used to calculate the moisture content.

Ash. The gelatin was ignited in a platinum dish, then heated at 550° to constant weight, and the residue weighed as ash.

Shrinkage temperature. Observations were made on water-saturated samples, freely suspended in toluene. Preliminary determinations were carried out at a temperature rise of 2°/min. The final determination was carried out at a 1° rise/min., when the temperature was within 10° of the preliminary value. A maximum variation between readings of 1·5° was obtained, and the true value taken as the average of three determinations.

RESULTS

A summary of the analytical data is given in Table 1. The weight recovery is the weight of amino acids recovered from 100 g. of protein multiplied by the average residue weight and divided by the average amino acid weight. The average residue weight is equal to the average amino acid weight less the weight of one molecule of water. The nitrogen recoveries of these materials were slightly lower than average.

As with collagens and gelatins from other vertebrates, traces of cysteic acid were found in the hydrolysates. This presumably originated from small amounts of cystine.

DISCUSSION

Throughout this section the figures refer to g./100 g. of dry, ash-free protein.

Analysis of chicken-leg tendon by microbiological and ion-exchange techniques

The results in Table 1 for chicken-leg tendon show reasonable agreement with those of Neuman (1949) for the same material analysed by microbiological methods. Large differences (greater than 1 in 20) between the two methods occur for tyrosine, methionine and histidine, where the absolute values are very small, and for lysine where Neuman's figures may include hydroxylysine. Alanine and serine show discrepancies of about 1 in 6, and isoleucine 1 in 4. Similar differences between the techniques have been reported for other samples (Eastoe, 1955).

In view of the totally different techniques used, and the considerable difficulty of securing reliable results by microbiological assay, the general agreement must be regarded as very satisfactory.

Characteristics of the amino acid composition of chicken-leg tendon

The composition is very similar to mammalian collagen and gelatins, with the exception of a few amino acids (cf. Eastoe, 1955, for the mammalian figures). Although the hydroxyproline is the same as for mammalian material, the hydroxylysine content of 1·68 is extremely high, and is only approached by the figure of 1·5 for human tendon. The increase in hydroxyl groups due to hydroxylysine is offset by the decrease in serine. The serine content is lower than for either mammalian or fish materials (cf. Eastoe, 1955, 1957). This adjustment is not shown in the composition of human tendon. The value for lysine is the lowest so far encountered for mammalian collagens and gelatins, and is only partially compensated by the high hydroxylysine value. Other differences are not large enough to be significant.

Amino acid composition of amphibian- and reptile-skin gelatins

The proportions of certain amino acids in the amphibian-skin gelatin suggest that this material occupies an intermediate position between mammalian and fish materials. The comparison is complicated by the wide range of values for certain amino acids, given by the different groups of fish materials. The toad-skin gelatin shows considerable

Table 1. *Amino acid composition of gelatins and related analytical data*

G, g. of amino acid/100 g. of dry ash-free protein. *N*, Amino acid N as % of total N. *M*, Moles of amino acid/10^5 g. of dry ash-free protein. *Z*, weight of amino acids given on hydrolysis of 100 g. of protein (Chibnall, 1942).

	Crocodile-skin gelatin			Python-skin gelatin		
	G	*N*	*M*	*G*	*N*	*M*
Alanine	11·1	9·5	124·5	10·2	9·86	114·0
Glycine	26·5	27·6	354	21·6	24·9	288
Valine	1·98	1·29	16·9	2·15	1·59	18·4
Leucine	2·87	1·68	22·0	3·07	2·02	23·4
Isoleucine	1·63	0·95	12·4	1·40	0·92	10·6
Proline	16·1	10·7	139·7	12·5	9·41	108·8
Phenylalanine	3·20	1·48	19·4	2·14	1·12	13·0
Tyrosine	0·64	0·27	3·6	0·30	0·14	1·7
Serine*	4·83	3·51	46·0	4·18	3·43	39·7
Threonine*	2·87	1·84	24·1	1·94	1·41	16·3
Cystine	Trace	Trace	Trace	Trace	Trace	Trace
Methionine†	1·02	0·54	7·1	0·82	0·48	5·5
Arginine	9·42	16·6	54·2	7·92	15·7	45·5
Histidine	0·79	1·17	5·1	0·67	1·12	4·3
Lysine	4·04	4·23	27·7	3·67	4·35	25·1
Aspartic acid	6·62	3·79	49·7	5·83	3·78	43·8
Glutamic acid‡	11·69	6·07	79·5	8·35	4·91	56·9
Hydroxyproline	13·3	7·74	101·4	12·2	8·04	93·0
Hydroxylysine	0·87	0·82	5·4	0·59	0·63	3·7
Total	119·44	—	—	99·51	—	—
Amide N§	0·39	2·13	27·9	0·28	1·74	20·1
Total N	—	18·35	—	—	16·19	—
Mean residue wt.	—	91·40	—	—	91·18	—
Z	—	119·7	—	—	119·8	—
Recovery by wt. (%)	—	99·8	—	—	83·1	—
Recovery of N (%)	—	101·8	—	—	95·5	—
Moisture	—	14·4	—	—	19·7	—
Ash	—	2·1	—	—	6·5	—
Shrinkage temperature	—	59°	—	—	57°	—

	Toad-skin gelatin			Chicken-leg-tendon gelatin		
	G	*N*	*M*	*G*	*N*	*M*
Alanine	8·7	7·6	97·3	11·1	9·8	124·4
Glycine	24·4	23·2	298	27·0	28·2	359
Valine	2·55	1·69	21·8	2·52	1·69	21·5
Leucine	3·75	2·22	28·6	3·38	2·03	25·8
Isoleucine	1·83	1·08	13·9	1·55	0·93	11·8
Proline	12·5	8·48	108·9	16·2	11·05	140·5
Phenylalanine	3·17	1·49	19·2	2·56	1·22	15·5
Tyrosine	1·11	0·47	6·1	0·67	0·29	3·7
Serine*	6·92	5·12	65·8	3·26	2·44	31·0
Threonine*	3·12	2·04	26·2	2·46	1·62	20·7
Cystine	Trace	Trace	Trace	Trace	Trace	Trace
Methionine†	1·29	0·67	8·61	1·00	0·52	6·7
Arginine	8·42	15·2	48·9	8·46	15·3	48·6
Histidine	1·01	1·52	6·5	0·75	1·14	4·8
Lysine	4·23	4·51	28·9	3·01	3·24	20·6
Aspartic acid	7·25	4·25	54·5	6·94	4·10	52·1
Glutamic acid‡	11·36	6·02	77·3	11·82	6·32	80·3
Hydroxyproline	10·1	5·96	77·0	14·0	8·41	106·8
Hydroxylysine	0·70	1·09	4·31	1·68	1·63	10·4
Total	110·42	—	—	118·31	—	—
Amide N§	0·75	4·17	53·5	0·61	3·42	43·5
Total N	—	17·99	—	—	17·80	—
Mean residue wt.	—	93·33	—	—	91·13	—
Z	—	119·4	—	—	119·8	—
Recovery by wt. (%)	—	92·5	—	—	98·8	—
Recovery of N (%)	—	96·8	—	—	103·3	—
Moisture	—	4·2	—	—	14·4	—
Ash	—	4·9	—	—	4·6	—
Shrinkage temperature	—	54°	—	—	64°	—

* Corrected for decomposition during hydrolysis (Eastoe, 1955).
† Sum of methionine and methionine sulphoxide peaks. ‡ Corrected for decomposition on column.
§ Corrected for ammonia formed by decomposition of serine and threonine.

Table 2. *Shrinkage temperatures of collagens and their hydroxyproline and serine plus threonine contents*

Shrinkage temperature ranges for cold-water and warm-water fishes and mammals are taken from Gustavson (1953). For other references to sources of data see Discussion.

Collagen from		Shrinkage temp.	Range of shrinkage temp.	Content of hydroxyproline (g./100 g.)	Content of serine + threonine (g./100 g.)
Cold-water fishes	Halibut	40°	36–43°	9·1	9·3
	Cod	40		8·3	11·1
Warm-water fishes	Pike	55	53–57	7·9	—
	Shark	53		10·9	8·2
Amphibia	Toad	54		10·1	10·0
Reptiles	Python	57	57–59	12·2	6·1
	Crocodile	59		13·3	7·7
Birds	Chicken	64	59–67	14·0	5·7
Mammals	Ox	65		14·1	6·8
	Pig	62		13·5	6·3
	Whale	63		12·8	7·8
Hot-water fish	Lung fish	63		10·8	7·9

similarities to the main group of fish materials (cf. Eastoe, 1957) with respect to its content of hydroxyproline, proline, serine, threonine and, to some degree, methionine. The increased serine and threonine contents offset the loss of hydroxyl groups due to decreased hydroxyproline and hydroxylysine contents. The remaining amino acid contents are not sufficiently different from those for fish or mammalian materials to enable any distinctions to be drawn. The impurity level, indicated by the low weight recoveries, is rather higher than for many of the mammalian materials where nearer 100 % weight recoveries are obtained.

Crocodile-skin and python-skin gelatins are broadly similar to one another in composition, but appear to have much closer affinities to mammalian materials than the toad-skin gelatin. The serine contents are high, resembling fish materials, and this is the principal departure from a mammalian composition, since the methionine, threonine and hydroxyproline contents are not very different from those of mammalian materials.

Thermal stability of collagens and their hydroxyproline content

Gustavson's views (1953, 1955) on the connexion between the hydroxyproline content and thermal stability of collagen, as indicated by the shrinkage temperature, have been discussed by Eastoe (1957). Gustavson (1955) suggested that the stabilizing feature of collagen is the interchain-hydrogen bonding between the hydroxy groups of the hydroxyproline molecules and carbonyl groups of the adjacent chain or chains. The results of the present paper may be usefully incorporated with those already published to give a more complete picture. Table 2 includes data from Neuman (1949), Gustavson (1953, 1955), Eastoe (1955, 1957) and Dr A. Courts (private communication).

From Table 2 it can be seen that the increase in shrinkage temperature is very approximately associated with an increase in hydroxyproline content, but the exceptions are sufficient to throw doubt on the hypothesis. If the toad and python materials are corrected for low weight recoveries, the amounts of the amino acids under discussion become very close to those of shark and crocodile gelatin respectively. The shrinkage temperature appears also to be in roughly the same order as maximum environmental temperature, and here lung fish fits well into the scheme. The low hydroxyproline content of lung-fish skin, and the somewhat low values for whale skin and pike skin compared with members of the same shrinkage temperature group, suggests an adaptation to life in a different medium. It is not clear whether the physical environment or the available diet has produced, in the course of time, the changes observed.

The serine plus threonine contents appear to decrease in a rather irregular manner with increase in shrinkage temperature. The sum of threonine, serine and hydroxyproline throughout the series examined is approximately constant.

A start has been made on the study of the amino acid composition of invertebrate materials possessing collagen-like X-ray-diffraction patterns. Watson & Smith (1956) have shown that earthworm cuticle contains amino acids with non-polar side chains in amounts similar to collagens. The most striking features of the composition of this material are the extremely high hydroxyproline and the very low proline values.

SUMMARY

1. The amino acid composition of gelatins from two reptiles, an amphibian and a bird, has been determined by ion-exchange chromatography.

2. The agreement in values for gelatin from chicken-leg tendon by both microbiological and ion-exchange methods is very good except for alanine, serine and isoleucine.

3. Chicken-leg-tendon gelatin is similar in composition to mammalian materials. The composition of toad-skin gelatin resembles certain fish gelatins more closely than mammalian materials, and the reverse is true for the two reptile gelatins.

4. The relationship between shrinkage temperature and hydroxyproline and serine plus threonine contents was studied. A very approximate relationship appears to exist between hydroxyproline content and shrinkage temperature.

The author is very grateful to Dr G. R. Tristram, Biochemistry Department, St Andrews, for the amphibian and avian materials, and to Dr A. Courts, of the British Gelatine and Glue Research Association, for the determination of the shrinkage temperature values of the crocodile and python skins.

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The Metabolism *in vitro* of Tissue Slices from Rats given Triethyltin Compounds

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A detailed account of the signs of the acute and chronic toxicity of alkyltin compounds in animals has been given by Stoner, Barnes & Duff (1955), who concluded that the main site of action of trialkyltin compounds appears to be in the central nervous system. Histological studies have shown that rats fed on a diet containing triethyltin hydroxide develop a specific lesion of the white matter of the brain and spinal cord (Magee, Stoner & Barnes, 1957). The lesion is characterized by an increase in the fluid content of the white matter without any apparent damage to the cells. A second characteristic is the reversibility of the lesion and recovery of animals from chronic poisoning when triethyltin is no longer administered.

The present work is an attempt to elucidate the reason for the selective action of triethyltin on the central nervous system *in vivo*, since previous biochemical studies showed that rat-liver mitochondrial preparations were highly sensitive to triethyltin (Aldridge & Cremer, 1955) and that the site of action was on the oxidative-phosphorylation mechanism.

This paper describes the respiratory activity of rat-tissue slices in the presence of triethyltin. Of the tissues studied, only brain slices are sensitive to triethyltin and the altered metabolic activity is paralleled by the concentration of triethyltin in the slice whether it is added *in vitro* or administered to the rat before removal of the brain.

A preliminary account of the work has already appeared (Cremer, 1956).

MATERIALS AND METHODS

Tissues. The tissues were from adult albino rats, of 170–200 g. body wt., which had been fed on M.R.C. diet no. 41 (Bruce & Parkes, 1949) except where otherwise stated. In experiments where triethyltin was added to the diet triethyltin hydroxide in arachis oil was mixed with powdered M.R.C. diet no. 41. Food consumption was recorded and paired feeding carried out on control rats.

Slices of brain cortex, liver, kidney and heart were cut with a Stadie–Riggs type of tissue slicer (Stadie & Riggs, 1944). Brain subcortical white matter was prepared according to McIlwain (personal communication). Diaphragms and pieces of washed small intestine were cut into segments. Tissue slices of 0·5–0·7 mm. thickness were quickly prepared at room temperature and placed directly in Warburg flasks. Dry weights were determined by removing the slices from the flasks at the end of the incubation period and heating at 110° for 24 hr.

Each flask contained 3 ml. of Krebs–Ringer phosphate solution (Umbreit, Burris & Stauffer, 1951), with glucose 0·011 M, tissue slice of 50–60 mg. wet wt. and 0·2 ml. of 20% (w/v) KOH in the centre well for absorption of CO_2. The manometers were placed in a water bath at 37°, gassed for 5 min. with 100% O_2, equilibrated for a further 10 min. and O_2 uptake was measured at intervals up to 75 min. Q_{O_2} is expressed as µl. of O_2/mg. dry wt. of tissue/hr. Variations in this general procedure are given in the legends of the particular experiments.

Lactic acid was estimated by the technique of Barker & Summerson (1941) and pyruvic acid by the method of Friedmann & Haugen (1943) with the modification for increasing the sensitivity previously described (Aldridge & Cremer, 1955).

Glucose was estimated by the method of Nelson (1944), but the copper reagent used was that described by Somogyi (1945). The glucose uptake was calculated as the amount of glucose which disappeared from the medium during incubation.

Special chemicals. Glucose, lithium lactate, ethylenediaminetetra-acetic acid disodium salt (EDTA), dithiocarbazone (dithizone; British Drug Houses Ltd., Poole), sodium pyruvate (Roche Products Ltd., Welwyn) and iodoacetic acid (L. Light and Co., Colnbrook) were used.

Inhibitor. A stock solution of triethyltin sulphate [$(Et_3Sn)_2SO_4$; 10 mg./ml., $3{\cdot}94 \times 10^{-2}$ M-triethyltin] was prepared as previously described (Aldridge & Cremer, 1955).

Estimation of triethyltin. A sample of tissue weighing between 0·4 and 1·0 g. wet wt. was homogenized in 5 ml. of water with a Nelco homogenizer, at 4000 rev./min. Water (10 ml.) was added to the homogenate, followed by 5 ml. of 10% (w/v) tartaric acid. The contents of the tube were mixed thoroughly and allowed to stand for 5 min. Perchloric acid (30%, v/v; 5 ml.) was added, the contents were again thoroughly mixed, then centrifuged for 10 min. at 3300 *g*. A 20 ml. sample of the supernatant was taken, neutralized with 5 N-NaOH (approx. 6 ml.) and 2 ml. of borate–EDTA buffer was added (19 g. of $Na_2B_4O_7,10H_2O$; 12 g. of boric acid; 4 g. of EDTA diluted to 1 l.; pH 8·4). The tubes were left to cool to room temperature. Chloroform (10 ml.) was added and the mixture extracted for 3 min. by plunging a mushroom-ended glass rod up and down. The aqueous layer was removed by suction and the $CHCl_3$ layer was cleared by centrifuging. The following procedures were carried out in a darkened room owing to the instability of the coloured complex to be formed. The $CHCl_3$ layer was transferred to a test tube (0·75 in. diam.), 3 ml. of borate–EDTA buffer was added followed by 1 ml. of dithizone reagent [0·04% (w/v) dithiocarbazone in $CHCl_3$], and the contents were mixed for 1 min. with a mushroom-ended glass rod. The aqueous layer was removed by suction and the $CHCl_3$ layer containing the triethyltin–dithizone complex was read against a dithizone control in 2 cm. cells at a wavelength of 610 mµ, a Unicam SP. 600 spectrophotometer being used. Two drops of acetic acid (AnalaR) were then added to each tube (triethyltin does not form a complex with dithizone under acid conditions) and readings taken again. The difference value of the two readings, giving the amount of dithizone used, was compared with the amount used by a standard concentration of triethyltin. The presence of EDTA has previously been shown to prevent interference from Fe, Co, Mn and Cu ions (Aldridge & Cremer, 1957). Recoveries of triethyltin, added to tissue homogenates *in vitro*, from rat brain, liver, kidney and blood, are given in Table 1.

RESULTS

Effect of triethyltin added in vitro

Tissue slices from normal rats were incubated in Krebs–Ringer phosphate solution in the presence of triethyltin. Concentrations of 3×10^{-7}–3×10^{-6} M appeared to affect only brain-cortical grey matter, subcortical white matter and spinal cord (Table 2). At higher concentrations several other tissues became sensitive to triethyltin, particularly kidney and diaphragm. At the lowest concentrations of triethyltin used there is a slight stimulation of O_2 uptake of brain slices and spinal cord. The concentration causing stimulation of brain cortex and subcortical white matter appeared to be highly critical, since in two other experiments with the same dilution of triethyltin either no effect or slight inhibition of O_2 uptake was observed.

A more constant feature of the effect of triethyltin is the lowered pyruvate and increased lactate levels, a phenomenon previously noted by Aldridge & Cremer (1955) in rat-brain brei preparations, for which no satisfactory explanation could be given. Stotz & Bessey (1942), studying the effect of thiamine deficiency in pigeons on the pyruvic and lactic acid levels of blood, showed that although the absolute values for each acid varied for the controls

Table 1. *Recovery of triethyltin added to rat tissues* in vitro

Recoveries are expressed as the mean percentage recovery with the standard error of the mean (S.E.M.) and the number of determinations (in parentheses).

	Range of total triethyltin added (µg.)					
	3–5		6–10		16	
	Average recovery (%)	S.E.M.	Average recovery (%)	S.E.M.	Average recovery (%)	S.E.M.
Brain	77	±1·54 (5)	75	±2·83 (8)	78	±3·97 (6)
Liver	76·6	±4·9 (4)	73·7	±2·74 (8)	79·1	±5·46 (6)
Kidney	82·9	±6·27 (5)	77	±1·93 (8)	79·8	±2·95 (6)
Blood	—	—	85	±2·88 (6)	88	±4·8 (4)

Table 2. *Effect of triethyltin added to rat-tissue slices* in vitro

For brain-cortex, liver and kidney slices the details were as described in Methods. Triethyltin, where present, was added before the addition of the slices. After incubation for 75 min., slices were removed for dry wt. determination, and 3 ml. of 18% (w/v) trichloroacetic acid (TCA) was added to the remaining flask contents. After centrifuging, lactic and pyruvic acids were determined in samples of the supernatant. For brain white matter, spinal cord, heart, small intestine and diaphragm flasks of 7 ml. total capacity were used. Flasks were prepared in pairs, each containing 1 ml. of Krebs–Ringer phosphate with glucose 0·011 M, triethyltin where indicated, and tissue samples of approx. 25 mg. wet wt. After incubation for 75 min. the tissue samples were removed and 1 ml. of 18% (w/v) TCA was added to each flask. The contents of the paired flasks were pooled for pyruvic and lactic acid determinations. Q_{O_2} is expressed as μl. of O_2/mg. dry wt./hr., lactic and pyruvic acids as μg. found in the total fluid volume after TCA addition and pooling of samples where indicated. Temp., 37°.

Tissue	Concn. of triethyltin (M)	Q_{O_2}	Lactic acid (μg.)	Pyruvic acid (μg.)	Lactic acid/ pyruvic acid
Brain cortex	Nil	11·5	236	12·5	18·9
	$2·8 \times 10^{-6}$	4·3	531	3·5	151
	$9·3 \times 10^{-7}$	9·0	405	3·8	106
	$3·1 \times 10^{-7}$	13·5	301	6·6	45·5
Brain white matter	Nil	5·9	296	12·6	23·5
	$1·3 \times 10^{-6}$	3·9	376	4·9	77
Spinal cord	Nil	4·05	90	5·3	17
	$2·6 \times 10^{-6}$	5·06	152	3·4	45
	$9·8 \times 10^{-7}$	4·8	113	3·1	36
Liver	Nil	6·2	58	8·3	7·0
	$1·5 \times 10^{-5}$	6·4	80	6·4	12·5
	$4·9 \times 10^{-6}$	7·1	79·5	9·8	8·1
Kidney	Nil	14·1	59·5	6·7	8·9
	$2·0 \times 10^{-5}$	11·2	145	3·9	36·5
	$6·6 \times 10^{-6}$	11·8	55·4	4·0	14
Heart	Nil	7·6	125	11	11·4
	$4·9 \times 10^{-6}$	8·3	160	13	12·3
Diaphragm	Nil	6·9	43	4	10·8
	$1·3 \times 10^{-5}$	6·5	111	2	55
	$4·4 \times 10^{-6}$	6·3	50	2·5	20
Small intestine	Nil	5·2	272	4	68
	$4·9 \times 10^{-6}$	4·7	302	5·6	54

Table 3. *Effect of triethyltin and potassium cyanide on aerobic and anaerobic glycolysis of brain and liver slices*

Each flask contained 3 ml. of Krebs–Ringer phosphate with 0·011 M-glucose and triethyltin or KCN as indicated. Average wet wt. of tissue slice was 56 mg. When KCN was added the 0·2 ml. of 20% (w/v) KOH normally used in the centre well was replaced by 0·2 ml. of a mixture of equal parts of N-KCN and N-KOH (Krebs, 1935). For aerobic experiments the gas phase was O_2 and for anaerobic experiments N_2 was used. After incubation for 75 min. at 37° slices were removed and 3 ml. of 18% (w/v) TCA was added. Samples of the flask contents were taken for lactic and pyruvic acid determinations. The values are expressed as total μg. of each acid/flask.

	Aerobic			Anaerobic	
Inhibitor concn. (M)	Q_{O_2}	Lactic acid (μg.)	Pyruvic acid (μg.)	Lactic acid (μg.)	Pyruvic acid (μg.)
		Brain-cortex slices			
Nil	12·4	186	11·4	253	1·5
Triethyltin ($1·3 \times 10^{-6}$)	7·4	440	5·5	126	<1
KCN ($1·1 \times 10^{-5}$)	3·7	575	1·9	305	<1
		Liver slices			
Nil	6·2	58	8·3	167	<1
Triethyltin ($1·3 \times 10^{-5}$)	6·4	80	6·4	167	<1
KCN ($3·3 \times 10^{-5}$)	3·9	196	2·4	266	1·3

from experiment to experiment, the ratio of lactic to pyruvic acid was constant, but in thiamine-deficient animals there was an abnormal ratio. A similar way of presenting the relationship of lactic to pyruvic acid in normal and triethyltin-treated slices has been adopted here, and for brain and spinal-cord slices this ratio is increased in the presence of triethyltin (Table 2).

The anaerobic glycolysis of normal brain slices was studied (Table 3). The levels of lactic acid were low compared with values obtained by McIlwain (1956) and earlier workers, but Beloff-Chain, Catanzaro, Chain, Masi & Pocchiari (1955), using [^{14}C]glucose, found that anaerobic glycolysis in rat-brain slices was low, only 7 % of the added glucose being utilized compared with 50 % under aerobic conditions.

The present results show that the ratio of lactic acid to pyruvic acid under anaerobic conditions was appreciably higher than in an aerobic system owing mainly to the low pyruvate level. The effect of triethyltin on brain slices respiring under aerobic conditions could be explained by this compound causing the system to become anaerobic. The effect of triethyltin on the activity of brain and liver slices under anaerobic conditions was examined (Table 3) and lactic acid production by brain slices only was found to be inhibited. Cyanide, which resembles triethyltin in increasing the lactic acid : pyruvic acid ratio under aerobic conditions, was without effect on anaerobic glycolysis. It has previously been shown that rat-brain lactic dehydrogenase is not inhibited by triethyltin (Aldridge & Cremer, 1955). The effect of iodoacetic acid on brain slices described by Heald (1953) was confirmed and the lowering of both pyruvate and lactate levels under aerobic conditions by this compound indicated that triethyltin does not act by inhibiting a specific enzyme of the glycolytic chain such as triose phosphate dehydrogenase. To determine the effect of triethyltin on the uptake of glucose from the medium by brain-cortex slices, the amount of tissue added to each flask was doubled. Results of typical experiments under aerobic and anaerobic conditions are given in Table 4. They show that in the aerobic system the uptake of glucose measured for the control could be accounted for as O_2 consumed and lactic acid found in the medium. In the presence of triethyltin the O_2 uptake was lowered but the glucose uptake was increased, and this increase could be accounted for as lactic acid. Under anaerobic conditions both the glucose uptake and the lactate level were lowered by triethyltin. Since triethyltin has been shown to be a potent inhibitor of oxidative phosphorylation by brain mitochondria (Aldridge, unpublished work), the altered metabolism of brain slices caused by this compound appears to be more likely due to alterations in their levels of adenosine phosphates and pyridine nucleotides than to direct inhibition of known enzymes.

Effect of transferring brain slices from a medium containing triethyltin to fresh medium. To determine whether the effect of triethyltin added to brain slices *in vitro* was reversible, slices were incubated in a medium containing triethyltin for 45 min., then transferred to fresh medium and incubated for a further 45 min., the O_2 uptake, pyruvate and lactate levels being determined for each period. Results from a typical experiment are given in Table 5,

Table 4. *Effect of triethyltin on glucose uptake by brain-cortex slices*

Each flask contained 3 ml. of Krebs–Ringer phosphate with 0·011 M-glucose and $2·2 \times 10^{-6}$ M-triethyltin where indicated. Average wet wt. of tissue slices was 137 mg. Values are expressed as μmoles/flask/75 min.

	Aerobic			Anaerobic	
	O_2 uptake (μmoles)	Lactic acid formed (μmoles)	Glucose utilized (μmoles)	Lactic acid formed (μmoles)	Glucose utilized (μmoles)
Control	14·9	5·0	5·0	7·2	3·5
Triethyltin	6·47	12·4	6·85	4·52	2·23

Table 5. *Effect of transferring brain-cortex slices from Ringer medium containing triethyltin to fresh Ringer medium*

Components of the flasks were as given in Methods with $6·9 \times 10^{-7}$ M-triethyltin where indicated. After incubation for 45 min. one slice from each pair was transferred to a flask containing 3 ml. of Krebs–Ringer phosphate with 0·011 M-glucose. After gassing for 4 min. with O_2, readings of O_2 uptake were taken up to 105 min. Slices were removed for dry wt. determination and 3 ml. of 18 % (w/v) TCA was added to each flask. Oxygen uptake is expressed as μl. of O_2/mg. dry wt.

System	O_2 uptake (25–45 min.)	Lactic acid/ pyruvic acid	O_2 uptake (85–105 min.)	Lactic acid/ pyruvic acid
Control (not transferred)	4·0	—	4·0	14·4
Triethyltin (not transferred)	3·3	—	1·5	94·0
Control (transferred)	4·7	23	3·3	23·0
Triethyltin (transferred)	3·7	68	1·5	57·0

which shows that the inhibition of O_2 consumption is not reversed by transferring the slices. Although the lactic acid:pyruvic acid ratio is less than in slices remaining in a medium containing triethyltin it is still higher than the control.

Distribution of triethyltin in rat tissues

The method for estimating triethyltin in biological material, described under Methods, appeared sufficiently reliable for it to be used in the present study to determine the pattern of distribution and approximate concentrations of triethyltin in the tissues and organs of rats given triethyltin, in an attempt to correlate the biologically effective concentrations *in vivo* and *in vitro*.

Rats placed on a diet containing 20 p.p.m. of triethyltin hydroxide for 5 days, followed by 10 p.p.m. for 12 weeks, were killed; various organs were removed and analysed individually for their triethyltin content. Results given in Table 6 show that although triethyltin could be detected in all tissues examined it was not evenly distributed, the highest concentrations being found in blood and liver. The concentrations of triethyltin found in spleen, kidney, heart and brain were lower. The compound does not accumulate in the central nervous system more than in other tissues.

Results given in Table 6 are mean values from three determinations expressed as μg. of triethyltin/g. wet wt. of tissue, correction being made for the percentage recoveries given in Table 1. The tissues were washed thoroughly before analysis and no allowance has been made for the blood content. From a sample of rat blood to which 26 μg. of triethyltin had been added, 23 μg. was recovered from the erythrocytes plus stroma and none was detected in the plasma. This pattern of distribution is different for rabbit blood where, in a similar experiment, 9 μg. was recovered from the erythrocyte fraction and 17 μg. from the plasma.

Activity of tissue slices from rats given triethyltin

Rats given a diet containing triethyltin do not gain in weight, owing primarily to a low intake of food (Stoner *et al.* 1955). In the present study, pair-fed controls have been used. Rats fed with triethyltin which were used for the experiments described in Table 7 were from the same group of rats used for the distribution data given in Table 6. For comparative purposes the activities of slices prepared from normal and pair-fed controls are also given (Table 7). Histological examination of the brain and spinal cord of the rats on a diet containing triethyltin showed that the lesion of the white matter previously described by Magee *et al.* (1957) was well established. Brain-cortex slices from rats fed with triethyltin showed an altered metabolism *in vitro*, whereas liver and kidney slices had an activity similar to those prepared from pair-fed control animals. Magee *et al.* (1957) showed that the lesion of the white matter had disappeared in rats which had been placed again on a normal diet for 4 months. The activity of brain-cortex slices prepared from two rats again put on a triethyltin-free diet for 12 days was normal (Table 7), and no triethyltin could be detected in the various organs analysed.

Activity of tissue slices from rats given a single dose of triethyltin. Since the brain-cortex slices with an altered metabolism *in vitro*, recorded in Table 7, were prepared from rats in which a marked lesion of

Table 6. *Concentration of triethyltin in tissues of three rats given a diet containing the compound*

Diet contained 20 p.p.m. of triethyltin hydroxide for 5 days followed by 10 p.p.m. for 12 weeks. Average total consumption was 11 mg. of triethyltin hydroxide/rat. For analytical procedure see text and Methods. Total blood vol. has been taken as 7% and skeletal muscle as 50% of body wt. Average wt. of rats when killed was 230 g.

Tissue	Mean total wet wt. (g.)	Triethyltin: Concn. (μg./g. wet wt., corr.)	Triethyltin: Total (μg./tissue)
Blood	—	17·6	283·0
Brain	1·78	2·9	5·2
Heart	0·82	5·2	4·3
Spleen	0·68	5·5	3·8
Liver	9·90	19·8	196·0
Kidney	1·77	6·4	11·3
Skeletal muscle	—	1·46	206·0
		Total	709·6

Table 7. *Activity of tissue slices from rats given a diet containing triethyltin*

For details see text and Table 2. Values are averages for three rats in each group.

Type of rat	Brain cortex: Q_{O_2}	Brain cortex: Lactic acid/pyruvic acid	Liver: Q_{O_2}	Liver: Lactic acid/pyruvic acid	Kidney: Q_{O_2}	Kidney: Lactic acid/pyruvic acid
Normal	10·2	27	6·0	7·2	16	7·1
Pair-fed control	10·5	24	6·5	16·2	14·8	9·9
Fed with triethyltin	6·6	82	6·0	11·2	14·4	6·9
Placed back on normal diet for 12 days	12·3	19	7·4	4·0	17·8	4·7

the white matter had developed, the activity of slices from rats given single doses of triethyltin was examined. Rats given an intraperitoneal injection of triethyltin sulphate (10 mg./kg.) were killed after 2 hr. and slices from various tissues were prepared. Their activities are given in Table 8. The dose used was lethal; rats not killed at 2 hr. died within 2–3 days. Again only nervous tissue was affected.

Uptake of triethyltin by brain and liver slices

Table 9 shows the amount of triethyltin found in brain-cortex and liver slices after they had been incubated in the Krebs–Ringer phosphate medium containing triethyltin. Under the conditions of the experiments the ratio of the volume of the medium to the wet weight of tissue was approximately 50. With both brain and liver slices, when triethyltin was added at two different concentrations in the medium the percentage taken up by the slices was the same. Similar results were obtained for brain white matter and kidney slices. If the triethyltin found in the slice after incubation was calculated as the molar concentration in the tissue fluid of the slice (taking the tissue fluid as 80% of the wet weight and making no allowance for any swelling of the slice in the medium) there was a concentration of triethyltin approximately 17 times greater in the slice than in the medium added at the beginning of the experiment. This factor has been used to calculate the amount present in slices from a known concentration added in the medium where the concentrations of triethyltin were too low to come within the sensitivity of the analytical method. The effect of several known metabolic inhibitors and lowered temperature were examined (Table 9) in an attempt to determine whether the uptake of triethyltin by tissue slices required energy or whether it was due to physical-adsorption processes. Only a lowered temperature caused a reduction in the amount of triethyltin found in the slice.

Comparison between dose, symptoms and activity of brain slices

Rats were given intraperitoneal injections of triethyltin sulphate in the doses stated in Table 10, and killed 2 hr. later. The brains were rapidly removed, the activity of slices prepared from one half was determined and triethyltin analysis carried out on the other half. Triethyltin was also

Table 8. *Activity of tissue slices from rats given a single dose of triethyltin*

For details see text and Table 2. Values are averages for two rats in each group.

	Normal		Triethyltin injected intraperitoneally	
Tissue	Q_{O_2}	Lactic acid/ pyruvic acid	Q_{O_2}	Lactic acid/ pyruvic acid
Brain cortex	11·5	19·0	5·2	108·0
Brain white matter	5·9	23·5	3·6	97·0
Liver	6·2	7·0	6·2	6·6
Kidney	14·1	8·9	15·7	9·8

Table 9. *Distribution of triethyltin between Krebs–Ringer phosphate and tissue slices and the effect of metabolic inhibitors*

Flasks were prepared in duplicate; each contained 3 ml. of Krebs–Ringer phosphate with 0·011 M-glucose. Triethyltin, and metabolic inhibitors where indicated, were added together before the addition of the tissue slices. The centre well contained 0·2 ml. of 20% KOH, except where KCN was used, when it contained 0·2 ml. of a mixture of 10 ml. of N-KCN and 1 ml. of N-KOH (Krebs, 1935). After incubating for 60 min. at 37° (except where otherwise stated) the slices were removed, rinsed quickly with water, blotted, homogenized in pairs and analysed for triethyltin. Total triethyltin added was determined for each experiment. *R* is the quotient: molar concn. of triethyltin in the tissue fluid (80% of tissue wet wt.) of the slices/molar concn. added in the medium.

Inhibitor concn. (M)	Concn. of triethyltin added (10^{-5} M)	Concn. of triethyltin found in slices (10^{-4} M)			
		Brain	*R*	Liver	*R*
—	5·3	10·4	20	8·8	16·5
—	1·77	2·7	15	2·5	14
Iodoacetic acid (10^{-2})	7·25	12·5	17	14·5	20
Malonate (10^{-2})	6·2	11·7	19	14·7	24
KCN (10^{-3})	6·95	11·8	17	12·2	17·5
Temp. lowered to 12°	7·75	6·7	8·7	—	—
Temp. lowered to 12°	8·46	—	—	7·25	8·6

determined in blood, liver and kidney samples. The results given in Table 10 show there is an agreement between the dose given, the intensity of the signs of poisoning, the amount of triethyltin present in the tissues and the lowered metabolic activity of brain-cortex slices.

Comparison of the activity of tissue slices with triethyltin added in vitro *and* in vivo. The values given in Table 11 were obtained in an attempt to correlate the activity of tissue slices prepared from rats given triethyltin with normal slices to which triethyltin had been added. The concentrations of triethyltin added to tissue slices *in vitro* have been calculated as described above (Table 9). The concentrations *in vivo* after intraperitoneal injection or feeding have been calculated as the molar concentration of triethyltin, assuming all the triethyltin found in the brain on analysis to be in the tissue fluid. Results of the activity of brain-cortex slices show that the effect of triethyltin on O_2 uptake and the lactic acid:pyruvic acid ratio was similar whether the triethyltin was added *in vitro* or given to the rat *in vivo*. Triethyltin had no effect on liver slices. The data were obtained from rats given intraperitoneal injections of triethyltin sulphate as described in Tables 8 and 10, except where otherwise stated.

Effect of 2:4-*dinitrophenol on brain slices in the presence of triethyltin*

Many workers have stimulated brain-cortex slices with 2:4-dinitrophenol (DNP), and Ghosh & Quastel (1954) in particular have shown that the stimulated portion of the O_2 consumption is more sensitive to certain narcotics than is the unstimulated portion. The effect of triethyltin on DNP stimulation of brain slices has been determined; the results are given in Table 12. In the experiments recorded, DNP was added to brain slices after they had been respiring in the presence of triethyltin so that a comparison could be made with slices prepared from rats injected with triethyltin.

Experiments in which slices were first stimulated with $2{\cdot}7 \times 10^{-5}$M-DNP for 30 min. and then had 8×10^{-7}M-triethyltin added, showed that the stimulation of O_2 uptake was abolished by triethyltin. Since brain slices in the presence of

Table 10. *Comparison of dose, symptoms and activity of brain slices*

For details see text and Table 2. Values are averages for two rats in each group. Symbols are used to indicate the presence and intensity of the signs of poisoning in the rats at the time of killing: −, rats appeared to behave no differently from the controls; +, rats showed minimum movement and ruffled hair; + +, rats showed also signs of muscular weakness, particularly of the hind limbs; + + +, rats were prostrate.

Dose of triethyltin (mg./kg.)	Symptoms	Triethyltin (μg./g. or ml.)				Brain cortex			
		Brain	Liver	Kidney	Blood	Q_{O_2}	Lactic acid	Pyruvic acid	Lactic acid/ pyruvic acid
Nil	−	0	0	0	0	12·6	186	11·4	16
2	−	2	12·2	3·8	10·8	12·9	206	8·9	23
4	+	1·6	10·6	4·5	14·6	14·7	223	8·3	27
8	+ +	3·3	17·6	6·3	48	10·3	389	5·4	72
12	+ + +	4·2	25	9·0	95	5·9	368	4·0	92

Table 11. *Comparison of the activity of slices with triethyltin added* in vitro *and* in vivo

For details of triethyltin concentrations see text.

Tissue	Concn. of triethyltin in tissue fluid (M)		Q_{O_2}	Lactic acid/ pyruvic acid
	Added *in vitro*	Intraperitoneal injection *in vivo*		
Brain cortex	—	—	12·6	16
	$4{\cdot}8 \times 10^{-5}$	—	4·3	151
	$5{\cdot}3 \times 10^{-6}$	—	13·5	45
	—	$2{\cdot}5 \times 10^{-5}$	5·9	92
	—	$1{\cdot}7 \times 10^{-5}$*	6·6	82
	—	$9{\cdot}7 \times 10^{-6}$	14·7	27
Liver	—	—	6·2	7·0
	$2{\cdot}5 \times 10^{-4}$	—	6·4	12·5
	$8{\cdot}3 \times 10^{-5}$	—	7·1	8·1
	—	$1{\cdot}5 \times 10^{-4}$	6·2	6·6
	—	$1{\cdot}2 \times 10^{-4}$*	6·0	11·2

* Rats were fed with triethyltin; see Tables 6 and 7.

Table 12. *Effect of 2:4-dinitrophenol on rat-brain-cortex slices in the presence of triethyltin*

Flask contents were as described in Table 2. A volume (0·1 ml.) of 2:4-dinitrophenol ($8·35 \times 10^{-5}$M) was added from the side arm after incubation for 45 min. and readings of O_2 uptake were continued until 85 min.

Concn. of triethyltin in tissue fluid (10^{-5}M)			O_2 uptake (μl./mg. dry wt.)		Change in O_2 uptake
Added *in vitro*	Intraperitoneal injection *in vivo*	DNP added	15–45 min.	55–85 min.	(%)
—	—	–	6·2	6·4	+3
—	—	+	4·5	7·2	+60
1·1	—	–	5·6	3·0	−40
1·1	—	+	4·3	2·3	−47
—	2·5	–	3·2	2·8	−12
—	2·5	+	3·6	2·7	−25

triethyltin could not be stimulated by DNP, and since in fact the inhibition of O_2 uptake increased with time to the same extent as with triethyltin alone, it seemed of interest to see whether brain slices prepared from rats injected with triethyltin could be stimulated by DNP. Rats were killed 2 hr. after an intraperitoneal injection of triethyltin (12 mg./kg.), and brain slices were prepared in the usual way and treated as described in Table 12. The molar concentration of triethyltin in the brain-tissue fluid of the injected rats was calculated as described for Table 11 by using the value of triethyltin found in the brain by analysis given in Table 10. Similar results to those given in Table 12 were obtained with slices of brain white matter; again neither brain slices with triethyltin added *in vitro* nor slices prepared from rats injected with triethyltin could be stimulated by DNP. All attempts to stimulate rat-liver slices with DNP failed. Results of other workers have shown small and variable stimulation (McCord, 1934; Muntwyler, 1935; Thomson, 1950), and Niemeyer & Figueroa (1955) have demonstrated that the initial glycogen content of liver slices influences their response to DNP.

DISCUSSION

The finding from experiments in which triethyltin was added to rat-tissue slices *in vitro* that only brain slices were sensitive to this compound was of particular interest, since previous work with rat-liver mitochondrial preparations had shown that triethyltin was a potent inhibitor of oxidative phosphorylation (Aldridge & Cremer, 1955). Aldridge (unpublished work) has since shown that triethyltin at low concentrations inhibits the oxidative phosphorylation of rat-brain mitochondria. A difference in sensitivity to certain drugs between whole-cell and fractionated-cell preparations is well known. Nevertheless, a difference of over 20-fold in the sensitivity to triethyltin between rat-brain and -liver slices remains striking, since both tissues are inhibited by similar concentrations when mitochondrial preparations are used.

Wenner & Weinhouse (1956) have confirmed that liver slices utilize only a small percentage of added glucose compared with non-hepatic tissues, and Gey (1956) has recently shown that rat liver contains ten times as much glucose as brain per g. of tissue. Brain tissue is known to be highly dependent on glucose so that a possible explanation of the sensitivity of this tissue to triethyltin could be that this compound in some way prevents glucose, added externally to brain slices, from reaching metabolic sites within the slice. However, this seems unlikely from the results given in Table 4 where, under aerobic conditions, triethyltin in fact increased the glucose uptake of brain-cortex slices. The simultaneous increase in lactic acid indicates that triethyltin interferes with the final oxidative pathway rather than the initial phosphorylation of glucose.

Since tissue slices other than brain were unaffected by triethyltin at low concentrations it seemed possible that under the experimental conditions the actions of drugs on certain tissues were not readily detectable. However, 5×10^{-5}M-KCN (Table 3) altered the metabolic activity of both brain and liver slices in a similar manner, and recently Coke & Mija (1956) have shown that rat-liver and -jejunum slices are markedly inhibited by certain antispasmodic drugs in conditions identical with those used in the present work. Webb & Elliott (1951), also using similar experimental conditions, found that pentobarbital inhibited the oxygen uptake and acid production of both brain and kidney slices.

Since measurements of the distribution of triethyltin between medium and tissue slices showed that similar amounts were taken up by brain, liver and kidney slices, there appeared to be an equal chance of inhibiting each type of tissue. Results from a single experiment, in which triethyltin was determined in nuclear, mitochondrial and micro-

somal plus supernatant fractions prepared by differential centrifuging techniques (Aldridge, 1957) from the liver of a rat injected with triethyltin, showed that triethyltin was present in each fraction. However, it is not possible to assess how far redistribution of triethyltin occurs during the preparation, and therefore the distribution pattern of triethyltin within different tissues *in vivo* remains obscure.

The mechanism whereby triethyltin exerts its effect on brain slices is still far from clear. The present results appear only to confirm the conclusions reached in previous work *in vitro* with rat-liver mitochondria and brain brei preparations (Aldridge & Cremer, 1955). Although at low concentrations triethyltin caused a slight increase in oxygen uptake of brain slices the results were variable and a more common feature was inhibition. It seems unlikely therefore that triethyltin acts like DNP, particularly since DNP added to brain slices in the presence of triethyltin did not show an increased oxygen uptake. Furthermore, V. H. Parker (unpublished work) has found that triethyltin prevents the stimulation of adenosine triphosphatase activity by DNP.

Triethyltin appears to be a stable substance in animal tissues when compared with many other drugs, thus rendering it a convenient compound with which to relate action to concentration. Distribution data given in Table 6 show that the selective action of triethyltin on the central nervous system cannot readily be explained by the compound becoming concentrated in this tissue, although from the chemical properties of triethyltin one might expect it to be preferentially distributed in lipid material, in which the central nervous system is particularly rich. However, within 1–2 hr. after an intraperitoneal injection triethyltin can be detected in the brain in sufficient amounts to account for its effect on metabolic activities. Triethyltin is concentrated by brain slices when this substance is added to the medium, and, as shown in Table 11, it has been possible to remove brains from rats obviously ill from triethyltin poisoning to determine the concentration of triethyltin in the whole brain, and to demonstrate an altered metabolic activity *in vitro* almost identical with that of normal slices to which triethyltin has been added to give a final concentration in the slice similar to that in the brains of the injected rats. Although the correlation between activity and concentrations *in vitro* and *in vivo* appears to be a direct one, it has not been ascertained where the triethyltin is located or how much is active, and calculations have only been made assuming it to be entirely in the total tissue fluid. Nevertheless, such a close agreement between the active concentrations is of interest, since similar comparisons with other drugs have sometimes shown wide discrepancies (Buchel & McIlwain, 1950; Butler, 1950; Sutherland, Hine & Burbridge, 1956). With these other drugs, however, closer agreement between the active concentrations has been obtained when the concentration *in vitro* has been taken as that which inhibited the portion of brain-slice oxygen consumption stimulated electrically or by K^+ ions (Ghosh & Quastel, 1954; McIlwain, 1953), whereas triethyltin, at low concentrations, inhibits the unstimulated portion. The altered activity of brain slices from rats suffering from triethyltin poisoning was similar whether the rats had been maintained on a diet containing triethyltin and showed a marked oedema of the white matter, or whether they were killed 2 hr. after an intraperitoneal injection of triethyltin. Since slices of both grey and white matter were affected, although only white matter shows pathological damage, it is not clear whether the biochemical lesion observed is related to the pathological one, and work on high-energy phosphate levels and turnover rates is being done in these laboratories to help to elucidate the relationship, particularly since no cellular damage has been detected histologically. The present results appear to indicate that the altered activity of brain slices from triethyltin-treated rats is due directly to the presence and concentration of triethyltin in the tissue, thus causing a similar behaviour to that of normal slices with triethyltin added *in vitro*. The fact that DNP did not stimulate brain slices from triethyltin-injected animals would seem to support this idea.

The lowered oxygen consumption of brain slices prepared from rats given triethyltin is very striking, in some instances being as low as 50 % of the normal rate (Table 8). It is possible that brain metabolism *in vivo* is not lowered to this extent and that this low activity of brain slices is in some way accentuated by the technique used. Some support to this idea is given by a few (unpublished) results for brain slices, obtained with a different technique, devised by Rodnight & McIlwain (1954) and Huston & Martin (1955). Slices were suspended in a moist atmosphere on silver-gauze mats and oxygen consumption was recorded by the conventional Warburg method. Brain slices, examined in this way, prepared from triethyltin-injected rats, had rates of oxygen consumption similar to those of control slices, although when duplicate slices were placed in Krebs–Ringer phosphate solution they showed a marked inhibition of oxygen uptake as described above. The outstanding difference between the two results indicates the difficulty of comparing data from tissue slices *in vitro* with possible changes *in vivo*. Although both techniques have obvious limitations it still seems pertinent that, with the procedure used throughout the present study, brain slices removed

from rats given triethyltin show a lowered metabolism *in vitro*, whereas slices prepared from liver and kidney, which contain relatively high concentrations of triethyltin, do not differ from the controls. Many earlier attempts (summarized by Quastel, 1955) to prepare brain slices from animals treated with drugs, particularly narcotics, and to demonstrate an altered metabolism *in vitro* have failed. It therefore seems of special interest that a substance such as triethyltin can produce so striking an effect.

The following comparisons between the clinical symptoms and tissue-slice activity of triethyltin-poisoned rats can be made. In both instances triethyltin appears to act selectively on the central nervous system. When rats which have been fed on a diet containing triethyltin are again given a normal diet, the oedema of the white matter disappears and the activity of brain slices returns to a more normal level. Peruzzo & Forni (1953) have made similar comparisons with chlorpromazine. They also demonstrated that the oxygen consumption of brain slices only was inhibited, both when chlorpromazine was added *in vitro* or when slices were prepared from injected rats. A further similarity between triethyltin and chlorpromazine is that both inhibit oxidative phosphorylation of rat-liver mitochondria (Andrejew & Rosenberg, 1956; Berger, Strecker & Waelsh, 1956), whereas liver slices are unaffected. Both compounds *in vivo* have a tranquilizing effect on rats and bring about a marked lowering of body temperature (Stoner *et al.* 1955; Peruzzo & Forni, 1953). There is nothing known about the effect of repeated doses of chlorpromazine on brain structure.

SUMMARY

1. The effect of triethyltin on the respiratory activity of rat-tissue slices has been examined. Brain slices are the most strongly inhibited.

2. Brain slices prepared from rats given triethyltin show a lowered metabolism *in vitro*. Liver and kidney slices do not differ from controls.

3. Data on triethyltin distribution in the rats show that the highest concentrations are in erythrocytes and liver.

4. *In vitro*, brain, liver and kidney slices take up triethyltin from the medium to produce a concentration greater in the tissues than in the medium.

5. When the amount of triethyltin found in whole brain *in vivo* and in slices *in vitro* is expressed as the concentration in the tissue fluid, there is a close correlation between the concentrations which are effective *in vivo* and *in vitro*.

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Trace Elements in Human Tissue

1. A SEMI-QUANTITATIVE SPECTROGRAPHIC SURVEY

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It is now known that the human body contains, in addition to the major mineral cation constituents—sodium, potassium, magnesium, calcium and iron—another group of elements, whose quantitative levels are uncertain and whose functions are not completely understood. The term 'trace element' is usually applied to this group which, in extremely small quantities, is known to play a vital part in the metabolism of plants and animals. In this paper, however, the term trace element will be extended to include all those metals present in minute amounts in tissue, irrespective of metabolic function.

The realization of the essential function of these minor constituents in plant and animal growth has prompted more attention to their evaluation in recent years. Stiles (1946) has reviewed much of the literature on trace elements in plants and animals and the significance of these substances in relation to nutrition. The toxicological importance of certain metals has also stimulated research on this subject for health reasons, and methods have been devised for establishing the levels of many elements in plant, animal and food materials. This aspect of the subject has been comprehensively reviewed by Monier-Williams (1949).

Considerably less attention, however, has been devoted to the analysis of trace elements in animal and human tissues than to those of plants. Previous research on this subject has been reviewed by Smith, Yeager, Kaufman, Hovorka & Kinney (1951), Kehoe, Cholak & Story (1940*b*) and Davis & Loosli (1954).

With the advent of large-scale atomic-energy programmes in many countries and the use of radioactive substances in reactors, in research and as weapons, a new significance has been given to trace elements in living materials. It is inevitable that there will be a small escape of fission products and of materials with neutron-induced radioactivity into air and water supplies as a result of these programmes. In order to evaluate the hazard due to the metabolism of these substances it is necessary to obtain an estimate of the levels of the various stable elements which occur in the human body. This information has not been available hitherto except for the major constituents. The semi-quantitative spectrographic survey described has been carried out on behalf of the Medical Research Council's Committee on Protection against Ionizing Radiation in order to provide this information in respect of the stable trace elements normally present in the human body.

Comprehensive surveys have also been carried out recently by Tipton, Foland, Bobb & McCorkle (1953), Tipton, Steiner, Foland, Mueller & Stanley (1954), Butt, Nusbaum, Gilmour & Didio (1954) and Koch, Smith, Shimp & Connor (1956).

The present survey has been carried out with an emphasis on minimum manipulation before analysis. This seemed justified in a preliminary survey, even at the expense of sensitivity, in order to avoid the risk of contamination associated with chemical concentration methods.

EXPERIMENTAL

Reagents

Hydrochloric acid. The Polaritan Grade (Hopkin and Williams Ltd.) was redistilled in all-quartz apparatus. The middle fraction was collected and stored in a stoppered polythene bottle.

Nitric acid. A.R. grade HNO_3 was redistilled in all-quartz apparatus. The constant-boiling-mixture distillate was stored in a stoppered polythene bottle.

Acetone. A.R. grade was redistilled from a Pyrex apparatus. The middle fraction was collected and stored in a polythene bottle.

Distilled water. Water distilled from a Manesty 00BE still was redistilled from Pyrex glass and collected and stored in a polythene aspirator.

Chemicals for standard mixtures. Specpure (Johnson, Matthey and Co. Ltd.) chemicals were used or the metal oxide was prepared by dissolving H.S. grade (Johnson, Matthey) metal in purified HNO_3 in dishes of silica or platinum and heating to form the oxide.

Carbon electrodes. (Supplied by Charles H. Champion and Co. Ltd.; 5 mm. × 200 mm. solid pure.) Spectrographic examination of these electrodes under the conditions described below showed them to be free from the trace elements under investigation with the exception of boron (strong lines) and copper (weak lines).

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Preparation of standards for spectrography

Soft tissue. All standards and samples of tissue ash were ground in agate mortars with the precautions against contamination described below. A series of synthetic standards were prepared, based approximately on 'standard man' (Recommendations of the International Commission on Radiological Protection), and similar to those prepared by Tipton *et al.* (1953). The basis for the soft-tissue standards consisted of a mixture of the major inorganic components of soft tissue, i.e. KH_2PO_4, NaCl, MgO, $CaCO_3$ and Fe_2O_3. This basis or matrix of the major constituents, which were not estimated spectrographically, was common to all the soft-tissue standards. It was found to be free from spectrum lines of the trace elements under investigation.

A mixture containing 100 mg. of each metal was prepared from the following compounds: $AgNO_3$, Al_2O_3, $HAuCl_4$, H_3BO_3, $BaCO_3$, Bi_2O_3, CdO, Co_3O_4, Cr_2O_3, CuO, Li_2CO_3, Mn_3O_4, MoO_3, NiO, Nb_2O_5, PbO, RbCl, SiO_2, SnO_2, $SrCO_3$, TiO_2, V_2O_5, ZnO, ZrO_2. By dilution with the mixture of major soft-tissue constituents described above, standards were prepared, containing 1000, 400, 200, 100, 40, 20, 10, 4, 2, 1 p.p.m. of each metallic element.

Bone standard. Calcium phosphate as a basis or matrix for the bone standard was prepared from 'specpure' $CaCO_3$, purified HCl and A.R. grade $(NH_4)_2HPO_4$. The calcium phosphate was examined spectrographically and found to be free from lines of the trace elements under investigation.

Tissue specimens

An endeavour was made to obtain tissue specimens from persons as near normal as possible [cases from instantaneous accidental death found to be free from evidence of gross organic disease at autopsies performed at various hospitals in the British Isles (see acknowledgements)], since little is known about the relationship between disease and trace-element distribution (cf. Olson, Heggen, Edwards & Gorham, 1954). For purposes of comparison, samples were collected from children and from infants dying in the neonatal period. Samples were also obtained from cadavers with evidence of disease.

In an attempt to determine the influence of local geology on the distribution of trace elements, some specimens were collected from people who had lived in the north-east of Scotland (Old Red Sandstone) and in the Cornish peninsula (granite), but insufficient specimens were available to make a significant comparison. Information about age, sex, occupation, diagnosis and cause of death, duration of disease and details of medication with metals was recorded as routine at autopsy.

A special effort was made to obtain samples which had had minimum contact with the dissection knife. The specimens were placed in polythene bags in a waxed container and transported to the laboratory immediately. After refrigeration at $-15°$ until hardened, the tissues were dissected with a quartz knife, a portion being selected from the centre of the specimen. Tissue surfaces, possibly contaminated at the autopsy by instruments, dust or body fluids, were thus eliminated. Samples dried in dishes of fused silica at 100° for 12–15 hr. were weighed, transferred to a muffle furnace and ashed at 500° for 12–15 hr. or until a white ash was obtained. To eliminate contamination from the roof of the muffle furnace, a Vitreosil (silica) lining was fitted. The greatest care was exercised at all stages in order to reduce contamination from atmospheric particles. Filtration of the air supplied to the laboratory through Vokes filters was unsatisfactory because the amount of dust in the air entering was so high as to cause rapid exhaustion of these filters. The supply of air under pressure to the laboratory was blocked off and manipulation of the ash was carried out in a portable 'dry box' constructed of Perspex. This was maintained at a slight positive pressure, by passing in compressed air filtered through a column of glass wool 12 in. high. Spatulas ground from Perspex rod were used for scraping the ash on to glazed paper (Teledeltos paper, free from the trace elements under investigation; supplied by the courtesy of Wiggins Teape and Alex Pirie Sales Ltd.). The ash was then stored in polythene containers.

Spectrographic method

A semi-quantitative procedure using the cathode-layer d.c. arc was employed. Estimation of the trace elements was based on visual comparison of suitable selected spectral lines with corresponding ones on a standard plate prepared by examination of mixtures of known trace-element concentration.

Apparatus. Spectrograph, Hilger Medium Quartz; spectrograph slit, 10 μ; photographic plate, Kodak IIN, backed, 10 in. × 4 in. or 10 in. × 2 in. as required; distance of source from (*a*) masking screen, 26 cm., (*b*) slit of spectrograph, 100 cm.; arc gap, 8 mm.; mask gap, 3 mm.; electrodes: lower, cathode, carbon (see below); upper, anode, carbon rod; current, 9 A d.c. at 200 V; exposure times, 3 min.

Sample form and electrode loading. The ground tissue-ash or standard was mixed with an equal quantity of spectrographically pure carbon by grinding in an agate mortar. The mixture was tamped into a hole $\frac{1}{32}$ in. in diameter, 12 mm. deep, in the special carbon electrodes (Slatter & Stitch, 1955, 1956), by the techniques of Mitchell (1948). The electrodes were cut from the carbon rod supplied by Champion and Co. on a machine tool made for this purpose (Slatter & Stitch, 1955, 1956).

Procedure. An image of the discharge arc was focused on the masking screen of a Bausch and Lomb optical head, and light from the anode was masked by adjusting the screen. The light from the cathode layer was focused on the slit of the spectrograph. To use the benefits of a 3 min. exposure (averaging of random variation in emission due to variation of electric current, air currents, etc., differential volatilization of elements and reduction of timing errors), an adjustable rotating light sector was constructed. This was placed immediately in front of the slit of the spectrograph. A 30° opening passing one-twelfth of the total light was selected as giving a satisfactory exposure.

Photographic processing. The Kodak IIN plates were developed for 4 min. with rocking in Kodak D 19 b developer in total darkness, placed in a 2% acetic acid for 30 sec., fixed in Fixol (Johnson and Sons Ltd., Hendon, London, N.W. 4) (diluted 1:3) and washed in tap water for 30 min. and rinsed with distilled water.

Evaluation of plate. The intensity of the spectral lines was compared visually with the corresponding lines on the standard plate. This was carried out in a Judd Lewis Comparator or, preferably, a projection comparator.

Reproducibility of method. With each series of samples analysed, a standard mixture of trace elements was exposed on the spectrographic plate. The intensity of the spectral lines of the trace elements from this standard was checked against those of the original standard plate. The results for a number of such determinations are shown in Table 1.

Table 1. *Reproducibility of spectrographic method*

Soft-tissue standards were examined with separate photographic plates over a period of 2 years. Concentration of various elements was estimated in terms of an original standard plate: (*a*) range, (*b*) 'mean', (*c*) number of determinations.

Standard concn. (p.p.m.)		Element						
		Al	Au	Cd	Mn	Mo	Cu	Ti
20	(*a*)	20–40	—	20–40	10–30	—	15–40	—
	(*b*)	27	—	23·7	16·3	—	25	—
	(*c*)	8	—	8	8	—	8	—
100	(*a*)	100–150	70–150	40–150	70–200	70–150	70–100	70–200
	(*b*)	116·7	89·4	97·8	98·9	103·3	93·3	117·2
	(*c*)	18	18	18	18	18	18	18
200	(*a*)	200–300	150–200	150–200	200–300	200–200	100–200	200–400
	(*b*)	250	175	175	250	200	150	300
	(*c*)	2	2	2	2	2	2	2
400	(*a*)	400–400	300–400	200–300	300–700	400–400	300–400	400–400
	(*b*)	400	350	250	500	400	350	400
	(*c*)	2	2	2	2	2	2	2

Table 2. *Spectrum lines employed and corresponding minimum detectable concentration of element*

Element	Line	Sensitivity (μg./g. of ash) Soft tissue	Bone
Ag	5209·1	100	100
Al	3082·2	20	20
	3944·0	—	—
	3961·5	—	—
Au	2676·0	40	40
Be	3321·3	100	—
Bi	2898·0	400	1000
Cd	2288·0	20	4
	3261·1	—	—
Co	2411·6	40	40
	2424·9	—	—
Cr	3005·1	40	10
	3015·2	—	—
Cu	3247·5	10	10
	3274·0	—	—
Mn	2576·1	4	10
	2593·7	—	—
Mo	3132·6	20	10
Ni	3414·8	40	40
Pb	2833·1	100	40
Rb	6206·3	10	—
	6298·3	—	—
Sn	2421·1	100	40
	2429·5	—	—
Ti	2641·1	40	40
	2644·3	—	—
Zr	2567·6	200	400
	2568·9	—	—

Sensitivity of method. The sensitivity (minimum detectable concentration, expressed as μg./g. of ash) for the elements investigated is given in Table 2 for soft tissue and for bone. It was seldom possible to use the strongest lines of the elements to be determined, because of interference from the lines of iron and other matrix or trace elements. Those lines were selected for use where resolution from the lines of interfering elements was satisfactory or the line of the interfering element was too weak to interfere under the conditions employed.

RESULTS AND DISCUSSION

It cannot be overemphasized that the results obtained from this survey are only semiquantitative. The figures reported, both for ranges and mean, of the various concentrations of trace elements found are not intended to convey a definite numerical significance: they are orders of magnitude only. Nevertheless, such results show the organs in which certain elements occur and the approximate level of these elements. Comparisons between infants, children and adults have been made, and certain marked differences between these groups are at once apparent. No significant difference between sexes was detected, although Hartman, Laue, Neuberg, Rosenheim & Volmer (1934) reported slight differences between the sexes with respect to trace elements in liver. Similarly no obvious difference was apparent between levels or distribution of the elements in 'normal' adult persons and the specimens of pathological origin, although specific disease groups were not studied.

Aluminium, copper, manganese and rubidium were found regularly in most of the soft tissues examined, but rubidium was not detected in bone. These results are shown in Table 3. For the purpose of calculating a 'mean', values greater than 1000 p.p.m. (expressed as >1000 p.p.m.) were given a value of 2000 p.p.m. Values obviously much greater than 1000 p.p.m. were given the symbol $\geqslant 1000$ p.p.m. and 5000 was taken for calculation of the 'mean'. This assumption must be borne in mind in assessing the significance of the 'mean' values reported; they are not absolute values but may be regarded as approximate average levels.

Aluminium. There has been much controversy (e.g. McCollum, Rask & Becker, 1928; Kahlenberg & Closs, 1929) as to whether aluminium is a regular and normal constituent of human tissue. These results confirm those of most investigations that aluminium is regularly present.

Results obtained for this element must always be regarded with suspicion because of the ubiquitous occurrence of aluminium, as aluminosilicates, in dust. In this survey aluminium was detected in most specimens of soft tissue. The levels detected in bone were regularly very much lower than in soft tissue, and this is taken to indicate that values obtained for soft tissue were real and not resulting from contamination, since both bone and soft tissue suffered identical but minimal hazards of contamination. Most striking is the relatively high concentration of aluminium in adult lung (Fig. 1). This has been observed by other workers (e.g. Tipton *et al.* 1953, 1954; Koch *et al.* 1956). The marked increase in the concentration of this element in lung with age was demonstrated (Fig. 1) and is undoubtedly the result of inhaling large quantities of dust. An accumulation of this element with age is apparent also in liver and kidney (Fig. 1). The value of approximately 500 p.p.m. obtained for liver in this survey is comparable with that reported by Koch *et al.* (1956) (approx. 300 p.p.m.), but is higher than the figure of 62 p.p.m. reported by Tipton *et al.* (1953). Similarly, the value obtained for spleen (approx. 300 p.p.m.) agrees well with the value of 300 p.p.m. reported by Koch *et al.* (1956) and Tipton *et al.* (1954) (approx. 334 p.p.m.), but not with the value of 66 p.p.m. reported semiquantitatively by Tipton *et al.* (1953). The values obtained were generally higher than those reported by Kehoe, Cholak & Story (1940*a*, *b*).

Cadmium. The comparatively high levels of cadmium in the kidneys of adults are at once apparent from Fig. 1. Cadmium has been reported in bovine kidney (Malinga, 1941) and in the kidneys of other animals (Klein & Wichmann, 1945), and the concentration of this element in human kidney has been demonstrated by Tipton *et al.* (1953, 1954) and Koch *et al.* (1956).

Fig. 1 reveals that there is a marked difference in the levels of cadmium between the various age groups; the concentration increasing with age. Cadmium was regularly detected in the livers of adults.

Chromium. Chromium was not regularly detected in this investigation. The element was measured in specimens of liver (1), heart (1), thymus (2), cartilage (3) and testis (1) of some children. The element was not detected in any of the specimens of lung examined; this does not support

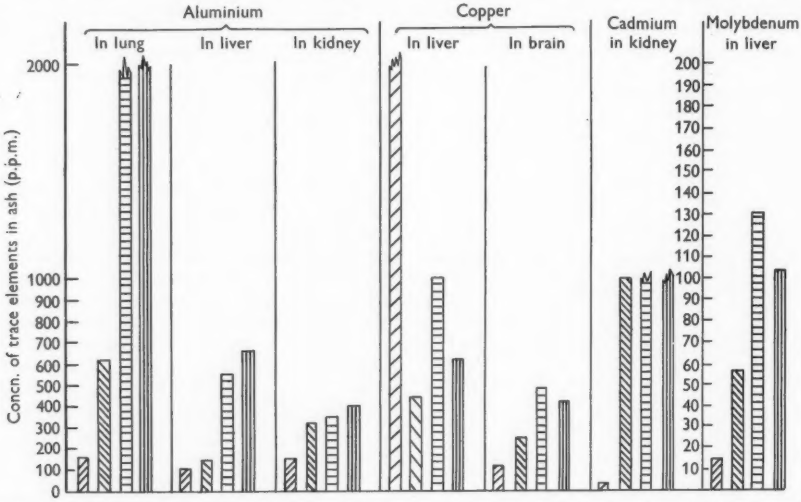

Fig. 1. Influence of age on the concentration of some trace elements in human tissues. ▨, 0–3 months; ▧, 1–12 years; ▤, material from normal adults 19–89 years; ▥, material from pathological adults, 18–89 years: the following diseases are represented in this group; carcinomas of kidney, rectum and bronchus, gastric ulcers, pneumonia, temporal glioma, hypertensive heart failure, coronary atheroma, thrombosis, mitral stenosis and acute stem-cell leukaemia. The values for individual disease groups were not found to differ significantly from those obtained from the normal adults. Broken columns at 1000 and 2000 p.p.m. indicate that calculated approximate average levels exceed these values respectively.

the findings of Ureone & Anders (1950), who reported 45 p.p.m. of chromium, calculated on the fresh tissue (approximately 4500 p.p.m. of ash).

Cobalt. This element was not detected in the tissues examined because the sensitivity for this element was not sufficiently good. Cobalt is now being sought in human soft tissues and bone, by radioactivation analysis.

Copper. The widespread occurrence of copper in tissues has been realized for several years. The highest concentrations of this element were observed in the livers of all groups, but especially so in those of foetus and infants. The copper of livers in this group was generally reported as $\gg 1000$ p.p.m., which probably means in excess of 5000 p.p.m. This high content of foetal and infant liver has been reported by several investigators, e.g. Sheldon & Ramage (1931), McElroy & Glass (1950) and Butt *et al.* (1954). It has been reported by Butt *et al.* (1954) that a similar disproportion between adults and the foetus occurs for iron and zinc, but not for manganese or lead.

The values for copper obtained in this survey agree well with those of other workers. Results for liver from adults (800 p.p.m. approx.) are within the range 300–910 μg./100 g. of fresh tissue reported by Tompsett (1935). The results agree also with those of Kehoe *et al.* (1940*b*), Butt *et al.* (1954) and Tipton *et al.* (1953, 1954). Results for copper in brain might suggest an increase in concentration with age contrasting with the decrease in liver after infancy (Fig. 1). Similarly the results obtained might suggest an increase in the concentration of copper in kidney with age. The values for adult brain are within the range noted by Tompsett (1935) and similar to those found by Bodansky (1921), Kehoe *et al.* (1940*b*) and Koch *et al.* (1956).

Table 3. *Approximate levels of some trace elements in the tissues of adults*

The figures (parts per million of the ashed tissue) given for range and mean are 'orders of magnitude' only.

	Aluminium			Copper		
Tissue	Range	Mean	No.	Range	Mean	No.
Adrenal	100–>1000	950	18	40–400	250	18
Bladder	<20–$\gg$1000	>1000	16	70–1000	250	15
Bone	<20–200	50	14	<10–70	20	14
Brain	—	—	—	70–1000	450	7
Cartilage	<20–>1000	650	14	40–700	175	14
Gall bladder	150–$\gg$1000	1000	11	200–>1000	750	11
Heart	<20–>1000	300	24	300–>1000	700	23
Kidney	<20–1000	400	26	150–>1000	550	26
Lung	1000–$\gg$1000	$\gg$1000	24	70–400	250	24
Liver	<20–>1000	500	25	100–>1000	800	25
Pancreas	20–>1000	600	17	70–>400	200	17
Prostate	100–>1000	1000	9	100–1000	250	9
Skeletal muscle	<20–>1000	550	17	40–700	150	18
Skin	40–$\gg$1000	1000	18	40–>1000	300	15
Spleen	<20–1000	300	22	40–>1000	200	23
Subcutaneous fat	40–$\gg$1000	>1000	20	70–700	250	19
Testis	20–1000	400	10	70–300	150	10
Thyroid	200–>1000	800	18	100–>1000	300	18

	Manganese			Rubidium		
Tissue	Range	Mean	No.	Range	Mean	No.
Adrenal	<4–70	35	18	100–>1000	400	17
Bladder	<4–70	20	15	70–>1000	400	14
Bone	<4–20	4	13	—	—	—
Brain	<4–40	15	7	70–150	100	6
Cartilage	<4–40	4	14	40–>1000	350	13
Gall bladder	<4–100	40	11	40–700	150	10
Heart	<4–20	10	23	40–>1000	450	23
Kidney	<4–200	60	26	100–>1000	550	25
Lung	<4–70	20	24	70–>1000	500	23
Liver	30–1000	150	25	100–>1000	500	24
Pancreas	20–200	100	17	70–>1000	400	16
Prostate	<4–30	10	9	40–150	100	8
Skeletal muscle	<4–30	<4	18	70–>1000	500	16
Skin	<4–20	<4	17	<10–>1000	250	16
Spleen	<4–300	30	23	40–>1000	500	22
Subcutaneous fat	<4–40	10	20	<10–>1000	400	20
Testis	<4–20	7	10	70–200	100	8
Thyroid	<4–70	20	18	40–>1000	300	17

The values obtained are somewhat lower than those reported by Tipton *et al.* (1954).

The values obtained for kidney in the adult group (550 p.p.m.) are higher than those reported by Koch *et al.* (1956; 200–300 p.p.m.) and Kehoe *et al.* (1940*a*). They are in agreement, however, with those obtained by Butt *et al.* (1954: 500 p.p.m. approx.). Similarly the values for copper in adult heart (700 p.p.m.) are slightly higher than reported by other workers but are in agreement with those of Butt *et al.* (1954: 600 p.p.m.).

Values obtained for copper in lung (250 p.p.m. approx.) are somewhat higher than the values (112 p.p.m.) reported by Tipton *et al.* (1953) and Kehoe *et al.* (1940*a*: 110 μg. of metal/100 g. of fresh tissue). Similarly the mean value obtained for spleen (200 p.p.m.) is higher than that reported (83 p.p.m.) by Tipton *et al.* (1953) and Kehoe *et al.* (1940*a*: 85 μg. of metal/100 g. of fresh tissue), but is lower than the figure reported by Tipton *et al.* (1954: 8·67 μg./g. calculated on the fresh substance).

Copper was irregularly detected in bone and at low concentration only. This is in agreement with the results of Tipton *et al.* (1953). Tompsett (1935) reported an average value of 15 p.p.m., which is in agreement with the results given above. Koch *et al.* (1956) quote a single value of approximately 20 p.p.m. for the copper content of bone. Kehoe *et al.* (1940*a*) reported figures of 0·410 mg./100 g. for rib and 1·19 mg./100 g. for long bone, calculated on the fresh substance.

Lead. The sensitivity for this element was not good and it was generally detected only in liver. The values obtained for lead in this organ (80 p.p.m.) are in reasonable agreement with those reported by Kehoe *et al.* (1940*a*: 130 p.p.m.). Lead was found in the bones of some adults but not in those of infants or children. It was detected in some specimens of heart, kidney, lung, pancreas and the thymus.

Manganese. The results obtained for manganese show the highest concentration to be generally in liver, although relatively high levels were observed in the infant group in thyroid and gall bladder. Insufficient numbers were analysed, however, to draw conclusions. The values obtained in the adult group for liver (approx. 150 p.p.m.), kidney (approx. 60 p.p.m.) and brain (approx. 15 p.p.m.) agree with the results of Reiman & Minot (1920), who reported results of approximately 170, 61 and 35 p.p.m., and with those of Kehoe *et al.* (1940*a*), who reported approximately 205, 60 and 30 p.p.m. respectively.

The concentration of manganese in brain appears to be somewhat lower in the infant and child than in the adult.

Molybdenum. This element was regularly detected in liver and kidney only. In liver, mean levels of about 120 p.p.m. were found in the adult, but values were some ten times less than this in the infant and half this value in the children 1–12 years old. This increase in concentration of molybdenum with age is of interest especially since this element is now known to be an essential cofactor of the xanthine oxidase system (Richert & Westerfield, 1953; De Renzo *et al.* 1953).

The values obtained for adult liver agree well with those reported by Butt *et al.* (1954: approx 141 p.p.m.) and Tipton *et al.* (1954: approx. 160 p.p.m.) but are higher than the mean figures earlier reported by Tipton *et al.* (1953: 12 p.p.m.) and by Koch *et al.* (1956: approx. 55 p.p.m.).

Nickel. This was detected in only two tissues in this survey, kidney (one specimen) and cartilage (one specimen). Nickel has been reported by some workers as regularly occurring in human tissues; that it was not generally detected in this survey was due presumably to the poor sensitivity (40 p.p.m.) for this element. Bertrand & Macheboeuf (1925) reported, however, that human and animal organs contained, as a rule, far less nickel than cobalt.

Rubidium. The accuracy for the determination of this element was poor. Nevertheless it was possible to demonstrate the widespread occurrence of rubidium at a comparatively high level in all the tissues examined except bone. This is in agreement with the results of Sheldon & Ramage (1931), who reported the widespread occurrence of rudibium in soft tissue and the absence of the element from bone. Of interest in connexion with the low values found for the infant group is the fact reported by Sheldon & Ramage (1931) that the only individual without rubidium was a 10 weeks' foetus.

The occurrence of rubidium in animal-eye lenses was reported by Sizeland (1952), and it was pointed out that the ratio of this element to the potassium present in the tissue was similar to the ratio of the natural abundances of these elements reported by Goldschmidt (1937).

Silver. This element was infrequently detected and only at comparatively low concentration. It was found in the lungs of three adults and in the aorta of one. Sheldon & Ramage 1931 detected faint traces of silver in five specimens of lung, but these workers found silver in every specimen of thyroid and tonsil, in 47% of the livers, 44% of the suprarenals and 36% of the lungs of eight individuals examined.

Similar irregularity of silver in biological material has been noted by Kehoe *et al.* (1940*b*), Dutoit & Zbinden (1930), Newell & McCollum (1931) and later workers.

Strontium and barium. These elements were not reported in the present survey, because the method was not sufficiently sensitive to detect them regu-

larly. Studies with radioactivation analysis (Sowden & Stitch, 1956, 1957) have, however, established the levels of both these elements in human bone.

Titanium. Titanium appeared more frequently and at a higher level in the lung of adults than in infants or children. The values obtained for adult lung (250 p.p.m.) agree well with the mean of 204 p.p.m. reported by Tipton *et al.* (1953). Titanium was also detected frequently in the skin and subcutaneous fat of adults. In this respect titanium follows aluminium, the highest levels of which were found in these situations.

SUMMARY

1. A method is described for the semiquantitative analysis of some trace elements in human soft tissue and bone, by cathode-layer arc spectrography.

2. Results are presented for the distribution of aluminium, cadmium, copper, manganese, molybdenum, lead and rubidium. Chromium, nickel, silver and titanium were detected infrequently in some organs.

3. Accumulation of some elements in certain organs was observed; relatively high concentrations of cadmium in kidney, copper and molybdenum in liver and aluminium in lung were observed regularly.

4. A comparison of the distribution and concentration of certain elements in the tissues of various age groups is presented and discussed. The concentrations of cadmium in kidney, of molybdenum in liver, of copper in brain and of aluminium in lung were shown to increase with age. In contrast the concentration of copper in liver was shown to decrease rapidly after birth.

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Trace Elements in Human Tissue

2. ESTIMATION OF THE CONCENTRATIONS OF STABLE STRONTIUM AND BARIUM IN HUMAN BONE

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The increasing development of nuclear reactors and weapons has necessitated some knowledge of the human metabolism of fission products. Strontium and barium have a particular significance in this respect, since they are among the most abundant of these products, have long half-lives, and are known to be deposited in bone.

The presence of barium and strontium in human tissues has been investigated by several workers with spectrographic techniques. Kunowski (1932) stated that there was no barium in normal human bones, and this was confirmed by Gerlach & Muller (1934), who could not detect barium in human bone although their method was sufficiently sensitive for its detection in several gallstones and faecal specimens. Arnolt (1941), by injecting stable barium chloride into experimental animals, showed that barium was retained chiefly in the liver, with some deposition in the lungs and spleen, but it was not found in the muscles, bones or kidneys. Timm (1932), however, using similar techniques, could detect traces of barium in animal bone, although he was not able to give quantitative details. Forbes, Cooper & Mitchell (1954) found that strontium was not present in the skeleton or soft tissues of the one human individual examined, at the limit of sensitivity of their spectrographic method (1 p.p.m. of ash), or by flame photometry (0·1 p.p.m.). Sheldon & Ramage (1931), investigating various human tissues spectrographically, detected strontium in some tissues but not in bone (limit of sensitivity 0·008 %). However, Gerlach & Muller (1934) maintained that strontium was present in normal and pathological bone in concentrations from 0·01 to 0·1 μg./g. of wet tissue, and Alexander, Nusbaum & MacDonald (1955) stated that the natural levels of stable strontium in the bones of mice, rats and guinea pigs are within the range 0·015–0·19 % of ash.

Asari (1950) estimated the strontium oxide content of 'protohistoric' and recent human bones and found the range to be 0·07–0·25 %. The average strontium concentration of 0·016 % for foetuses 10–18 cm. long and 0·24 % for male and female adults was given in a survey of the strontium content of human bone ash carried out by Hodges *et al.* (1950).

Tipton, Foland, Bobb & McCorkle (1953) stated that their spectrographic method was not suitable for the detection of strontium. Barium was not detected at the limit of sensitivity (200 p.p.m.). However, separate estimations of strontium in human bone by neutron-activation analysis, gave results ranging from 6 to 255 p.p.m. of ashed bone.

Results published for the spectrographic estimation of barium and strontium in bone show some discrepancy, and the reliability of this method for precise quantitative determinations is open to question. Standard analytical procedures for the estimation of these elements have been hampered by the difficulty of their separation from calcium. The advantages of neutron-activation analysis have been outlined previously (Smales, 1953, 1955; Brooksbank, Leddicote & Mahlmann, 1953; Jenkins & Smales, 1956). The sensitivity and precision of this technique render it particularly suitable for the determination of strontium and barium in bone. The errors and limitations inherent in the method have been described by several authors (e.g. Smales, 1953; Bowen & Dymond, 1955; Plumb & Lewis, 1955; Jenkins & Smales, 1956).

METHODS

The technique adopted for the present work was based on the method described by Harrison & Raymond (1955). It is dependent on the irradiation of ashed samples together with standards of strontium and barium salts under similar conditions. After irradiation the strontium and barium were separated from the ash and their concentrations estimated by a comparison of radioactivity with that of the standards. The addition of stable isotopes as carriers, subsequent to irradiation, facilitated the extraction of strontium and barium free from contamination by the radioactive isotopes of these elements. Preliminary investigations to determine the minimum weight for satisfactory recovery of inactive barium and strontium carrier showed that 50 mg. of each was sufficient to ensure an adequate chemical yield.

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It has been stated that the method has a high degree of sensitivity. Within the conditions of the experiment, the limit of detection was found to be 0·75 μg. for strontium and 1·0 μg. for barium. This assumes the minimal activity required for determination to be twice that of the background, allowing 2·25 hr. for the chemical separation. The limits of 0·04 μg. for strontium and 0·1 μg. for barium quoted by Bowen & Dymond (1955) could not be achieved under the conditions described.

Reagents

Standards. Johnson, Matthey and Co. Ltd. 'specpure' carbonates were used, the specified impurity being negligible.

Carriers. Standard solution of strontium and barium (50 mg./ml.) carbonates, A.R. grade, was used. Nitrates of potassium, copper, sodium, cadmium, bismuth, manganese, silver and lead in 10% (w/v) aq. solution were used. $(NH_4)_3PO_4$ was an aq. solution saturated at room temp.; $BaCl_2$ was (10% (w/v) aq. solution.

Other reagents. Na_2CO_3, A.R. grade, aq. solution saturated at room temp. K_2CrO_4, A.R. grade, aq. solution saturated at room temp. Ammonium acetate, A.R. grade, aq. solution saturated at room temp. Acetic acid, A.R. grade. $FeCl_3$, A.R. grade, 5% (w/v) aq. solution. HNO_3, Hopkin and Williams, C.P.R.; *d* 1·5 (fuming). HCl (2 N), A.R. grade.

Sample form and preparation

Specimens of various bones were collected from autopsies held in different parts of Britain. Material from 'normal' bodies was selected whenever possible (Stitch, 1957). Samples of bone were generally taken from the diaphyses of long bones or the shafts of ribs close to the costal angle. Strict precautions against contamination were observed; the material was transported in polythene bags, and adhering tissues were separated with a quartz knife. No chemical manipulation was permitted before activation. Specimens were dried in silica crucibles at 100° for 24 hr. and ashed in a muffle furnace at 500° for 24 hr. The bone ash was ground to a fine powder in an agate mortar and stored in polystyrene containers. This was carried out in a 'dry box' (Stitch, 1957), under pressure, so that the fine particles of dust present in the atmosphere were excluded. Instruments, after cleaning with acid, were rinsed with water redistilled from all-glass apparatus.

Activation of samples and standards

Two standard samples, each consisting of a weighed mixture of $SrCO_3$ (approx. 50 mg.) and $BaCO_3$ (approx. 100 mg.), and six samples of bone ash (approx. 150 mg.) each in small (1 cm. × 2·5 cm.) polystyrene containers, were irradiated simultaneously.

The samples were irradiated in a 3½ in. × 1 in. Al can placed in the 'belt stringer' of the Harwell reactor, B.E.P.O. The can was subjected to a neutron flux of 10^{12} neutrons/cm.²/sec. for 3 hr., the reactions being represented as follows:

$$^{138}Ba \rightarrow {}^{139}Ba \quad \text{(half-life, 1·4 hr.)}$$

$$^{86}Sr \rightarrow {}^{87}Sr \quad \text{(half-life, 2·8 hr.)}$$

The thermal neutron cross-sections for ^{138}Ba and for ^{86}Sr are 0·55 and 1·3 respectively. An activity of 40 mc/g. of barium and 12 mc/g. of strontium is obtained after 3 hr. irradiation in a neutron flux of 10^{12} neutrons/cm.²/sec.

After irradiation, the specimens were left for 30 min. before handling to allow the decay of intense radioactivity from isotopes of short half-life.

Radiochemical purification

The radiochemical purification scheme is shown in Fig. 1.

All chemical manipulations were carried out behind lead shielding of 2 in. thickness. Discarded radioactive solutions were stored behind concrete of 6 in. thickness during the analysis.

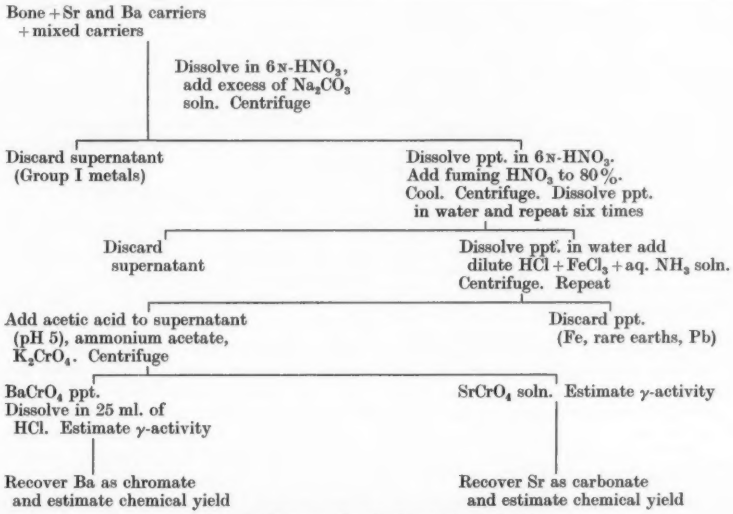
Bone + Sr and Ba carriers + mixed carriers
Dissolve in 6 N-HNO3, add excess of Na2CO3 soln. Centrifuge
Discard supernatant (Group I metals)
Dissolve ppt. in 6 N-HNO3. Add fuming HNO3 to 80%. Cool. Centrifuge. Dissolve ppt. in water and repeat six times
Discard supernatant
Dissolve ppt. in water add dilute HCl + FeCl3 + aq. NH3 soln. Centrifuge. Repeat
Add acetic acid to supernatant (pH 5), ammonium acetate, K2CrO4. Centrifuge
Discard ppt. (Fe, rare earths, Pb)
BaCrO4 ppt. Dissolve in 25 ml. of HCl. Estimate γ-activity
SrCrO4 soln. Estimate γ-activity
Recover Ba as chromate and estimate chemical yield
Recover Sr as carbonate and estimate chemical yield

Fig. 1. Scheme of radiochemical purification.

After activation the bone ash was weighed into numbered 40 ml. graduated centrifuge tubes. The standard samples were emptied into two 500 ml. volumetric flasks, the containers being washed four times with 2N-HCl to ensure the complete transference of the radioactive strontium and barium salts. The volumes were made up to 500 ml. with water. To obtain a level of activity suitable for counting, 1 ml. portions of the activated barium and strontium solutions were pipetted from each volumetric flask into two 40 ml. centrifuge tubes. Approx. 0·5 ml. of the solution of carriers containing potassium, copper, sodium, cadmium, bismuth, manganese, silver and lead were then added to each tube, and 3–4 ml. of 6N-HNO_3 to dissolve the ash. A volume (1 ml.) of the solution of stable barium and strontium carrier was added, and the active and stable barium and strontium were precipitated by the addition of excess of saturated aq. Na_2CO_3, then separated by centrifuging (approx. 1000 *g*; MSE major centrifuge), the supernatant liquid being discarded. The insoluble carbonates were dissolved in the minimum volume of conc. HNO_3, reprecipitated and the supernatant discarded as before. The precipitate was redissolved and the volume made up to 5 ml. with water; approx. 1 ml. of $(NH_4)_3PO_4$ carrier and 14 ml. of fuming HNO_3 were added (Willard & Goodspeed, 1936). After cooling in iced-water for 5 min., the tubes were centrifuged and the supernatants discarded. The nitrates were dissolved in 5 ml. of water and $(NH_4)_3PO_4$ and a further 14 ml. of fuming HNO_3 was added to reprecipitate them. Six precipitations were carried out in order to remove the calcium from the ashed samples. Ammonium phosphate, which was added to the first three precipitations, acted as a carrier for radioactive phosphorus. Calcium phosphate was not used since calcium would affect the chemical yield, but not the assay of radioactivity owing to the characteristics of the calcium isotopes produced during irradiation. After the final HNO_3 separation, the nitrates were dissolved in a minimum quantity of water, and $FeCl_3$ solution (approx. 0·1 ml.) and conc. HCl (approx. 0·1 ml.) were added. Ferric hydroxide was precipitated after adding aq. NH_3 soln., thereby scavenging traces of other metals. The precipitate was centrifuged and the supernatant decanted into a further series of centrifuge tubes. The remaining precipitate was dissolved in the minimum of 2N-HCl, and the iron scavenge repeated.

To the combined supernatants acetic acid and ammonium acetate were added until acid (pH approx. 5), together with 1 ml. of K_2CrO_4 to precipitate the barium. After heating in a boiling-water bath (2 min.) and centrifuging, the solutions of $SrCrO_4$ were decanted into numbered centrifuge tubes. The precipitated $BaCrO_4$ was dissolved in the minimum quantity of 2N-HCl and reprecipitated by excess of saturated ammonium acetate. This was repeated a third time, the final precipitate of $BaCrO_4$ being dissolved in 2N-HCl and the three supernatant strontium fractions being combined. In order to remove residual traces of ^{139}Ba from the strontium solutions, 0·5 ml. of 10% $BaCl_2$ was added as carrier and the precipitated $BaCrO_4$ was separated by centrifuging and discarded. Both strontium and barium fractions were made up to 25 ml. and transferred to separate polystyrene counting pots (9 cm. × 5 cm.).

Estimation of activity

The barium and strontium fractions were counted consecutively over a period (4 hr.) in a thallium-activated, NaI crystal-type γ-scintillation counter. A decay curve was plotted for each sample in order to check its purity (Fig. 2). Contaminated fractions which did not give a decay curve parallel to that of the standard were discarded.

After assay of radioactivity the solutions of barium and strontium were quantitatively transferred to weighed and numbered centrifuge tubes for estimation of the chemical yield. The barium was precipitated as $BaCrO_4$ with excess of ammonium acetate, and the strontium as $SrCO_3$ with excess of Na_2CO_3. The precipitates were centrifuged, washed twice with dilute aq. NH_3 soln. and once with acetone, and dried to constant weight.

The activity of each sample at a specific time was found from the decay curves, and was corrected for the paralysis time, background of the counter and for the overall chemical yield after radiochemical purification. The concentration of barium and strontium originally present in the bone ash was calculated by the equation:

$$M_x = \frac{R_x \times W_s \times Y_s \times f \times 10^6}{R_s \times W_x \times Y_x},$$

where M_x represents Ba (Sr) in bone ash (μg./g.); R_x, counts of Ba (Sr)/min., unknown fraction at time t; R_s, counts of Ba (Sr)/min., standard fraction at time t; Y_s, wt. (mg.) of Ba (Sr), recovered from standard fraction; Y_x, wt. (mg.) of Ba (Sr), recovered from unknown fraction; f, conversion factor for wt. of Ba (Sr), in carbonate irradiated as standard (Ba 0·696; Sr 0·594); W_s, wt. (mg.) of standard salt irradiated; W_x, wt. (mg.) of bone ash irradiated.

The average measurement of activity of two standard samples was used for comparison with the unknown samples. If the discrepancy between the two standard samples was greater than 5% the analysis was abandoned.

Reproducibility

Repeated determinations of the barium and strontium concentrations of five bone samples were made in order to estimate the reproducibility of results. Results given in Table 1 do not generally vary by more than 5% of the mean. For purposes of comparison, some of our material has been analysed for strontium by Dr B. A. Loveridge (Chemistry Division, Harwell) by neutron-activation analysis, but with ^{89}Sr (β-emitting isotope) and a more elaborate method of radiochemical purification. His results (Table 2) show remarkable agreement with results obtained by us in this laboratory.

RESULTS

Estimations were made of the concentrations of barium and strontium in 35 bone samples. Analyses were made in triplicate whenever sufficient material was available. The results for the various bone samples are presented in four groups according to age (Table 3). The levels of barium and strontium are expressed as μg./g. of ashed bone (p.p.m.).

It can be seen from Table 3 that the results for strontium in the two adult age groups (19–74 years) are higher than those for the two lower age groups (foetus and child 0–13 years). Statistical comparison of groups 1 and 2 with groups 3 and 4 shows that the level of strontium in the group 19–74 years is significantly higher than in the group 0–13 years

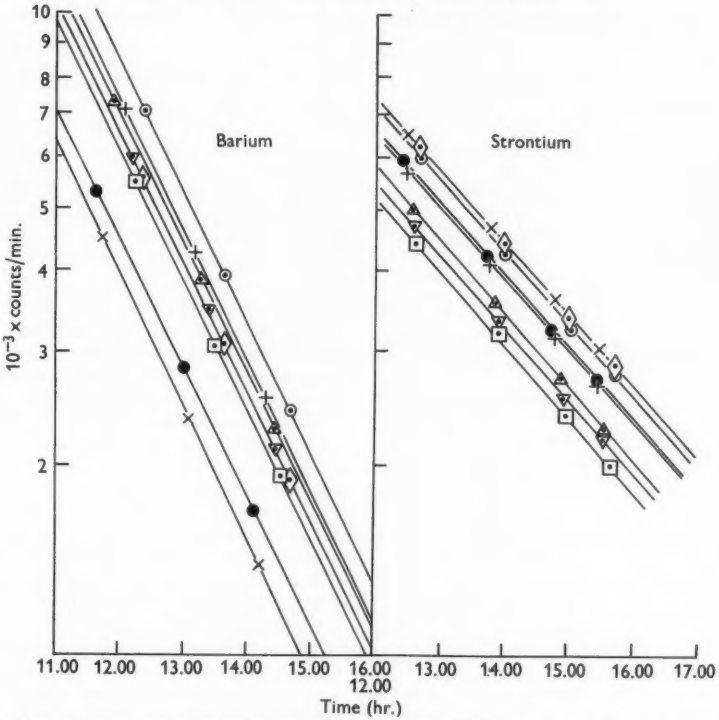

Fig. 2. Typical decay curves for barium and strontium after radiochemical separation from bone ash: ◇, standard 1; ⊙, standard 2; ●, ×, +, ▽, △ and ⊡, fractions 1–6 from bone ash.

Table 1. *Reproducibility of results of replicate determinations of barium and strontium in bone samples*

Case no.	Barium concn., mean and S.D. (p.p.m.)	No. of determinations	Strontium concn., mean and S.D. (p.p.m.)	No. of determinations
B37	11·4±0·6	8	144 ±4·5	8
B38	5·5±0·6	9	146 ±8·0	8
C16	4·7±0·2	5	90·4±5·0	6
C14	7·9±0·6	6	53·4±2·5	6
B16	4·0±0·9	5	85·0±2·0	6

Table 2. *Comparison of strontium values obtained for three bone samples, in different laboratories*

	Loveridge (see Methods)		Sowden & Stitch (present work)	
Case no.	Mean and S.D. (p.p.m.)	No. of determinations	Mean and S.D. (p.p.m.)	No. of determinations
M2	61±4	4	64±3	12
B37	143±5	4	144±4·5	8
B38	136±9	8	146±8	8

Table 3. *Concentration of stable barium and strontium in bones of human males and females*

Group	1	2	3	4
Age	0–3 months	1–13 years	19–33 years	33–74 years
No. in group ...	7	9	9	10
Barium				
Range (p.p.m.)	1·9–13·0	2·1–21·0	4·3–7·9	3·7–17·3
Mean (p.p.m.)	7·0	7·6	5·1	8·5
S.D.	±4·0	±7·0	±0·12	±4
Strontium				
Range (p.p.m.)	41·6–108·0	53–129	66·5–130	77–157
Mean (p.p.m.)	79·1	73·8	107	114
S.D.	±20	±26	±23	±28

Table 4. *Distribution of strontium (μg./g. of ash) in different bones of single individuals*

	Age of individual (years)			
	0	6	18	78
Rib	72	100	131	138
Tibia	66	98	114	179
Femur	66	91	127	157
Skull	75	—	126	180
Vertebrae	65	—	115	150
Sternum	—	—	113	135
Humerus	64	—	—	—
Pelvis	67	—	—	—
Clavicle	70	—	—	—
Fibula	67	—	—	—
Ulna	65	—	—	—

at P 0·01. No variability with age was noted in the barium level of these two groups. The greater range of barium results within an age group could not be related to any specific factor.

Analyses of several bones of the same individual were carried out on four specimens in the age range 0–78 years. Significant variability in the strontium (Table 3) or barium levels was not observed in the different bone samples of each individual examined, although higher values for the strontium content of the tibia and skull of one male, aged 78, were noted (Table 4).

The presence of barium and strontium was confirmed in all the bone samples analysed. There was no evidence of relationship between the concentrations of barium and strontium and the sex of the individual. No relationship of barium and strontium with disease was noted in the additional pathological cases investigated, e.g. cases of haemorrhage, ulcerative colitis, peritonitis, heart failure. Bone samples from patients with specific bone diseases have not been examined.

DISCUSSION

Most remarkable in this series of results is the constant presence of strontium and barium in all the bones investigated. Earlier work, which failed to determine these elements, was based on spectrographic methods. The poor sensitivity of this method probably accounts for the recorded absence of barium in bone.

We have found barium in bone in relatively lower concentration than strontium and the method of neutron-activation analysis described has made it possible to determine the levels with accuracy. The extensive physiological experiments which have been carried out with radioactive strontium tracers (Jowsey, Owen & Vaughan, 1953; Pecher, 1941; Kidman, Tutt & Vaughan, 1950) provide evidence that injected strontium is retained in bone in preference to other organs.

The conclusions of Hodges *et al.* (1950) are in agreement with certain points of our work, but the normal level of strontium recorded by these workers (0·024 %) is considerably higher than estimated in this Laboratory. The range of results for strontium in bone given by Tipton *et al.* (1953), from 6 to 255 p.p.m., is greater than that observed by other authors, and the average figure of 64 p.p.m. of strontium is lower. However, consideration must be given to the possibility of dietary variability in different geological locations, when comparing results from different countries. There is no obvious relationship between strontium and barium levels and locality within the British Isles, although specimens from areas where the soils are known to be comparatively rich in strontium have not been examined.

We are generally in agreement with Hodges *et al.* (1950) on the constancy of the strontium level in different bones of the same individual. MacDonald *et al.* (1951) produced data for the strontium content of the femurs and vertebrae of mice fed on diets containing strontium and noted that the strontium level was similar in the two bones. Experiments by Pecher (1941) with injected radioactive strontium suggest that strontium is preferentially deposited in certain bones; this author reported a higher activity in the vertebrae. Lisco, Finkel & Brues (1947), however, found that osteogenic sarcoma caused by the ionizing radiations of the strontium

were more common in the long bones. These observations may be explained by the realization that strontium is laid down in sites of active bone formation, which will vary with each experiment.

Hodges *et al.* (1950) found a difference in the strontium content of foetal and adult material. The foetal material was obtained from foetuses of length 10–18 cm. ($4\frac{1}{2}$–5 months intrauterine life), and these authors suggest that the lower values of foetal strontium may be due to the slight degree of calcification at this stage of development. Foetal material of this age was not examined by us. Comparison of our analyses of bones obtained from infants with those of bones from adults shows a slight but significant increase in the strontium content; this is not exhibited as a uniform increase throughout the period associated with bone calcification. The most marked increase appears to be in the first few months of life, with isolated results of high strontium content in the upper age group.

SUMMARY

1. A method is described for the determination of strontium and barium in human bone by radioactivation analysis.

2. Results of analyses of 35 bone samples, from normal persons of both sexes and different ages, are given. The concentrations of barium and strontium were found to be of the order of 7 and 100 μg./g. of ashed tissue respectively.

3. No relationship between sex or disease of individuals with strontium and barium concentration was noted. The concentration of strontium in the age group 0–13 years was significantly lower than that in the group 19–74 years.

4. No significant difference was found in the concentrations of strontium and barium in the various bones of those individuals examined.

5. Results obtained in this survey are discussed and compared with those of other workers.

We are indebted to Mrs A. Harrison for her help in the initiation of the programme; to Dr B. A. Loveridge (Chemistry Division, A.E.R.E., Harwell, Didcot, Berks.) for his co-operation in cross-checking the results on our samples, and to Miss Diana Clay for her invaluable technical assistance.

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Some Chemical Components of Mitochondria and Microsomes from Germinating Castor Beans

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Increasing interest in the biochemical activities of mitochondria from plants has made it desirable to have more knowledge of the chemical composition of such particles. We have, accordingly, extended our investigation of the highly active mitochondrial fraction from the endosperm of germinating castor bean (Beevers & Walker, 1956; Walker & Beevers, 1956) by studying some of the components of particulate preparations from this material. A considerable amount of analytical work has been done with particles of animal origin, but the data on biochemically active particles from plants is still rather meagre (see reviews by Goddard & Stafford (1954), and Hackett (1955). Previous work is discussed in papers by Martin & Morton (1956*a*, *b*), who have made thorough examinations of the chemical composition of mitochondria and microsomes derived from silver-beet petiole and from wheat roots. Another recent contribution provides data on the ribonucleic acid (RNA) and nitrogen contents of active mitochondria and microsomes from peanut (Kmetec & Newcomb (1956)).

In the next paper (Akazawa & Beevers, 1957), there are shown some very striking changes in the amount and intrinsic activity of the mitochondria derived from the endosperm as the castor-bean seedling grows to the 8 days stage, when the endosperm has been depleted. It was therefore of some interest to see if there were differences in the composition of the particles derived from seedlings of different ages. The information so obtained allows some conclusions to be drawn about the process of mitochondrial production and particularly about the decline in efficiency which characterizes the mitochondria from the ageing endosperm.

Osmotic phenomena are of some importance in determining the behaviour of plant mitochondria, as they are in those from animal tissues, and Laties (1953) and Farrant, Robertson & Wilkins (1953) have investigated aspects of the osmotic behaviour of plant mitochondria. These observations have been supplemented here by data on the percentage dry weight of mitochondrial particles and the changes in this value under a variety of experimental conditions.

MATERIALS AND METHODS

The culture of castor-bean seedlings and the preparation of mitochondrial suspensions were carried out as described previously (Beevers & Walker, 1956), with the following changes: (*a*) variety Cimmaron was used, since the supply of U.S. 74 failed; (*b*) blending for 15 sec. in a chilled blendor was substituted for manual grinding with pestle and mortar; (*c*) the phosphate concentration in the medium was reduced to 0·05 M. In this paper we refer to the washed mitochondrial suspension so obtained as 'crude mitochondria'. In preparing extracts a number of seeds, sufficient to give 40 g. of material, was used; the testa, root and shoot were discarded and in seedlings older than 6 days the cotyledons were also removed from the endosperm material. The crude mitochondria were in some cases separated into two components, which are designated as M_1 and M_2; the sediment M_1 was obtained by centrifuging for 10 min. at 1000 *g* and the resulting supernatant was exposed to 20000 *g* for 20 min. to provide sediment M_2. These fractions were resuspended in measured amounts of medium before analysis.

A further fraction, which will be designated 'microsomes', was obtained from the supernatant after removal of the mitochondria by centrifuging at 56000 *g* for 60 min. in a Spinco ultracentrifuge. The usual precautions were taken to ensure cold conditions (0–5°) during the various manipulations. The fractions were analysed individually for the components listed below, and for oxidative activity where this was appropriate.

Most of the results are expressed on a nitrogen basis to facilitate comparison with those obtained in the extensive work on fractions from liver. In addition, in order to arrive at a picture of the interrelationships between the various components during the cycle of growth and depletion of the endosperm, some of the results are expressed on a 'per bean' basis.

Q_{O_2} *measurements.* The oxidative activity of the mitochondrial fractions was measured with α-oxoglutarate (H.M. Chemical Co., Santa Monica, California) as substrate in the presence of the full complement of cofactors (Beevers & Walker, 1956) at 25°. A 60 min. oxidation period was allowed and portions were taken at the outset for determination of N.

Analytical. The phospholipid, acid-soluble phosphate ('nucleotide') and RNA contents of the mitochondrial fraction M_2 and the microsomes were determined by the procedure of Ogur & Rosen (1950). The residue after RNA extraction in $HClO_4$ was used for protein determination. In the RNA analyses, the factor 0·35 of Ogur & Rosen was employed to convert the E_{260} (absorption at 260 mμ) readings into RNA phosphorus.

Inorganic phosphate. This was determined by the Fiske & Subbarow (1925) method.

Total nitrogen. Sulphuric acid (5 N) was used to digest portions of the various fractions and the residue after RNA extraction, and the NH_3 was determined by nesslerization.

Percentage dry wt. of the particles. After centrifuging, a sample of about 70 mg. fresh wt. was transferred to a small tared bottle and the weight of material determined, before and after drying overnight at 100° (Bartley & Davies, 1954); dry wt. (%) is given by (dry wt./wet wt.) × 100.

RESULTS

By differential centrifuging (see Methods) the crude mitochondrial fraction was separated into two components, M_1 and M_2. The results in Table 1 show that almost all of the nitrogen of the original crude suspension was recovered in the two sediments, but that the total O_2 uptake of the two fractions was considerably less than the original. Almost all of the oxidative activity which was recovered was concentrated in M_2, and since this fraction contained about 60% of the original nitrogen, the Q_{O_2} was higher than that of the original crude suspension. The addition of M_1 to M_2 did not repair the loss in total activity, and apparently some irreparable damage attends the separation of the two components. Although the loss in total activity offsets to some extent the gain in specific activity, it is clearly of advantage to use the 'purer' M_2 for comparative chemical analyses; for other purposes the use of the crude mitochondria might be preferred.

The results of analyses of the M_2 fraction are given in Table 2, with values from the original crude suspension for comparison. It will be seen that although about one-half of the acid-soluble phosphate and RNA are not found in M_2, the contents relative to the nitrogen content have increased as a result of the separation.

Changes in the composition of mitochondria and microsomes

Figs. 1 and 2 summarize a series of analyses on the M_2 fraction and the microsomes respectively from the endosperms of seedlings from 3 to 7 days old. The lower graph (*B*) in each shows the course of the changes in phospholipid and RNA phosphorus and of acid-soluble phosphate relative to the protein-nitrogen content of the particles, while in the upper curves (*A*) the changes in the absolute levels per individual bean are shown. Up to the fifth day there is a progressive increase in the total amount of mitochondrial protein and subsequently an equally striking decline in this quantity. Accompanying these changes there are parallel changes in phospholipid and RNA. There is, in fact, a relationship between the amounts of RNA and phospholipid on the one hand and the amount of protein nitrogen on the other which is characteristic of the mitochondria of the endosperm regardless of the stage of development of the seedling.

The changes in acid-soluble phosphate, on the contrary, show that at least after the 4 days stage the mitochondria can not be regarded as having a characteristic level of this component. The amount of acid-soluble phosphate increases until the fourth day, but thereafter no extra acid-soluble phosphate accumulates and its concentration, whether expressed 'per bean' or in terms of protein N, shows

Table 1. *Fractionation of mitochondrial suspension*

Fractions M_1 and M_2 were suspended individually in sucrose–phosphate medium so that 1 ml. of this suspension corresponded to 1 ml. of the original suspension; substrate was α-oxoglutaric acid (0·01 M). q_{O_2}: μl. of O_2 absorbed/mg. of N/hr.

	O_2 uptake μl./30 min. Expt.		Total nitrogen mg./ml. Expt.		q_{O_2} Expt.	
	(*a*)	(*b*)	(*a*)	(*b*)	(*a*)	(*b*)
Crude mitochondrial suspension (1 ml.)	116·0	190·0	1·81	1·98	128·0	192·0
M_1 (1000 *g*/10 min.)	9·3	11·2	0·745	0·725	25·0	30·9
M_2 (20 000 *g*/20 min.)	66·5	119·0	0·955	1·120	140·0	213·0
$(M_1 + M_2)$/crude × 100	65·0	68·8	94·0	93·0	—	—

Table 2. *Chemical analysis of the original crude mitochondrial suspension and the purified mitochondria (M_2)*

(*a*) and (*b*) are separate preparations.

		Acid-soluble phosphate		RNA P		
		(μg./ml.)	(μg./mg. of protein N)	(μg./ml.)	(μg./mg. of protein N)	Protein N (mg./ml.)
Crude mitochondria	(*a*)	1140	400	39·8	14·0	2·85
	(*b*)	1180	437	47·3	17·5	2·70
M_2	(*a*)	500	650	20·6	26·9	0·77
	(*b*)	760	670	26·5	23·5	1·13

a sharp decline. We attach significance to this change in attempting to explain the striking decrease in the efficiency of the mitochondria which begins at about the 5 days stage (Akazawa & Beevers, 1957).

The total amount of microsomal protein, in contrast with that of the mitochondria, does not increase during the early stages; a slow decline in protein occurs to the 6 days stage and a sharper fall thereafter. The RNA and phospholipid show parallel changes but the microsomal protein declines more rapidly than these components during the later stages, so that the curves for RNA and phospholipid/mg. of protein nitrogen show a tendency to rise with time. The acid-soluble phosphate is again apparently the first of the various components lost from the microsomes during depletion of the endosperm; the amount relative to the protein nitrogen reaches a very low value after 6–7 days.

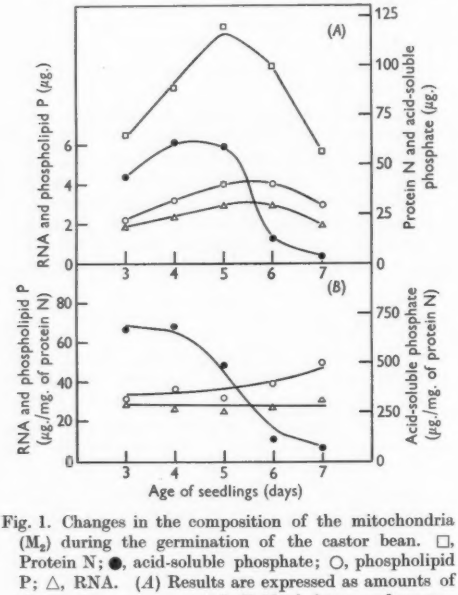
(A)
(B)
RNA and phospholipid P (μg.)
Protein N and acid-soluble phosphate (μg.)
RNA and phospholipid P (μg./mg. of protein N)
Acid-soluble phosphate (μg./mg. of protein N)
Age of seedlings (days)

Fig. 1. Changes in the composition of the mitochondria (M_2) during the germination of the castor bean. □, Protein N; ●, acid-soluble phosphate; ○, phospholipid P; △, RNA. (*A*) Results are expressed as amounts of the various components/individual bean endosperm; (*B*) components are expressed as amounts/unit of protein N.

It will be noted from Figs. 1 and 2 that the absolute levels of the various components are very different in the microsomes and mitochondria. For the purpose of comparing composition more closely, the data of Table 3 may be used. The 4 days stage was used because (*a*) all the components in M_2 increase together up to this point, and (*b*) M_2 still shows high oxidative and phosphorylating ability. There is about 50 % more nitrogen in the mitochondria than in the microsomes. The most striking difference is in the specific content of RNA; there is a fivefold superiority in favour of the microsomes. The acid-soluble phosphate content of the M_2 is, on the other hand, twice as great as in the microsomes. The phospholipid content of the mitochondria is roughly two-thirds of that of the microsomes.

Water content of castor-bean mitochondria and its experimental control

Table 4 shows the results of dry-weight determinations on crude mitochondria and on M_1 and M_2 fractions. Under the conditions of isolation the particles from castor beans have a considerably lower water content than those from other tissues

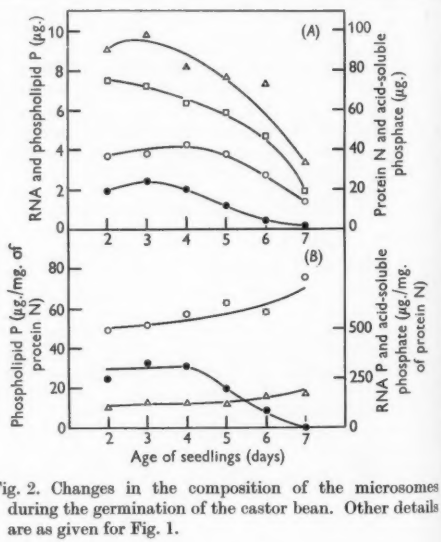
(A)
(B)
RNA and phospholipid P (μg.)
Protein N and acid-soluble phosphate (μg.)
Phospholipid P (μg./mg. of protein N)
RNA P and acid-soluble phosphate (μg./mg. of protein N)
Age of seedlings (days)

Fig. 2. Changes in the composition of the microsomes during the germination of the castor bean. Other details are as given for Fig. 1.

Table 3. *Chemical composition of mitochondria (M_2) and microsomes from 4 days seedlings*

	Phospholipid P (μg./mg. of protein N)	Acid-soluble phosphate (μg./mg. of protein N)	RNA P (μg./mg. of protein N)	Protein N (mg./g. of bean)
Mitochondria	36·9	685·0	26·5	0·220
Microsomes	57·0	310·0	134·0	0·148

Table 4. *Dry weight (%) of crude and purified mitochondria*

Age (days) ...	3	4	5	6
Crude mitochondria	30·9	31·1	28·3	25·0
M_2	—	25·8	25·9	—
M_1	—	27·0	—	—

Table 5. *Changes in dry weight (%) induced by exposure to media of different tonicities*

In III (*a*) and (*b*) refer to separate experiments.

	I	II	III	
Tonicity of medium in original suspension (M) ...	0·02	0·25	0·5	
			(*a*)	(*b*)
Treatment		Dry wt. (%) of particles		
None	18·9	24·2	29·5	29·0
0·02 M-Sucrose (30 min.)	17·5	17·3	19·7	—
0·25 M-Sucrose (30 min.)	—	—	—	25·4
0·5 M-Sucrose (30 min.)	28·2	27·6	30·6	30·1
0·8 M-Sucrose (30 min.)	—	—	—	35·0

which have been described (MacFarlane & Spencer, 1953; Bartley & Davies, 1954). After the fourth day there is a progressive increase in the water content.

Changes in the water content can be readily induced by exposing the particles to media of different tonicities, as shown in Table 5, column III. This table also includes data on mitochondria prepared in the usual way except that the washing and final suspension were in media of lower sucrose content (columns I and II). It will be seen that the dry weight of the resulting mitochondria varies with the concentration of the suspending medium. When these mitochondria with different water contents were subsequently exposed to media of different tonicities for 30 min. there were marked changes in weight, which seem to be explicable on simple osmotic considerations. Thus the water content of mitochondria as normally prepared (column III) was much increased by exposure to hypotonic solution (0·02 M) and to a lesser extent by 0·25 M-sucrose. In 0·8 M-sucrose there was a loss of water from the particles. If the mitochondria were allowed to remain in contact with their respective suspending media there was little further change in water content during a 30 min. period; equilibrium had apparently been reached in each instance during the washing, centrifuging and suspension in the cold.

Mitochondria, after treatment with 0·02 M-sucrose ('swollen mitochondria'), lost water on being exposed to 0·5 M-sucrose; apparently the changes in water content are reversible. The mitochondria from 0·25 M-sucrose are particularly interesting because they have initially an intermediate water content, and this can be increased by 0·02 M-sucrose and decreased by 0·5 M-sucrose. None of the following appeared to influence the swelling of normal mitochondria when they were exposed to media of lower tonicity: temperature (0–30°); 2:4-dinitrophenol (0·002 M); ethylenediaminetetraacetic acid (0·001 M); calcium chloride (0·01 M). In addition, neither the provision of adenosine triphosphate (1 mg./ml.) nor of conditions optimum for oxidative phosphorylation (Akazawa & Beevers, 1957) appeared to affect the swelling and shrinking of washed mitochondrial suspensions.

DISCUSSION

From the data presented here it is clear that the composition of the particles from the endosperm of the 4 days castor bean is not widely different from that of particles of animal origin which have been more fully examined (see, for example, Lindberg & Ernster (1954), Hogeboom & Schneider (1955). The washed mitochondria have a relatively high level of acid-soluble phosphate and also contain some RNA. The most striking difference in the two kinds of particles examined is in the RNA content; the microsomes have roughly five times as much RNA phosphorus/mg. of protein nitrogen as the mitochondria. Although we have no data at present which would allow us to ascribe the presence of RNA in the mitochondria to a small microsomal contamination, this must remain a possibility.

In so far as our results can be compared to those of Kmetec & Newcomb (1956) it would appear that the RNA content of the castor-bean microsomes is considerably higher than those from peanut. One important difference in the two preparations should be noted, however: the peanut particles were obtained at much lower centrifugal forces, and thus the separation of the microsomal particles from the castor bean may have been more effective. Although the relative RNA contents of the 'mitochondrial'

and 'microsomal' fractions reported by Stafford (1951) are closer to those now described for castor-bean particles, the differences in preparative procedure should be noted here also.

Martin & Morton (1956*a*) provide data and rigid tests for the reliability of the procedures employed, which show that in silver-beet petiole the RNA content of the microsomes is not nearly so widely different from that of the mitochondria as it is in the particles of animal origin, and draw attention to this difference. They point out, however, that the metabolic status of the tissue may be an important consideration, and they have provided data from germinating-wheat roots in which the RNA content of the microsomes is considerably higher (Martin & Morton, 1956*b*). In the castor-bean endosperm, a highly active material, the RNA level in the microsomes is not only five times as great as that in the mitochondria, but the absolute levels observed (on a protein-nitrogen basis) are close to those observed by Palade & Siekewitz (1956) for liver microsomes.

The day-to-day changes in the composition of the mitochondria have been compared with those occurring in the microsomes. No allowance has been made in this or in the succeeding paper (Akazawa & Beevers, 1957) for possible differences in the degree of completion with which the particles can be extracted and recovered from tissues of different ages by the standard procedure employed. Particular attention has been drawn to the striking changes in the acid-soluble phosphate content of the mitochondria, changes which do not follow those of the other components examined. The content of 6 days beans is only one-sixth of that at 4–5 days. In the microsomes also this component is the first to decline sharply.

The water content of the mitochondria is relatively low at all stages of development and regardless of the extent of swelling. Values of between 19 and 35 % dry weight are to be compared with those of sheep-kidney mitochondria [8–20 % dry weight (Bartley & Davies, 1954)] and of rat liver [11–22 % dry weight (MacFarlane & Spencer, 1953)]. Experiments with mitochondria from 4 days beans have shown that exposure to media of different tonicities results in rapid changes in the water content, pointing to a further similarity between mitochondria from animals and plants; a similarity which had in fact been inferred earlier from other kinds of experiments (Laties, 1953; and Farrant *et al.* 1953).

SUMMARY

1. Castor-bean mitochondria and microsomes prepared by differential centrifuging of homogenates in sucrose–phosphate have been analysed for ribonucleic acid (RNA), acid-soluble phosphate, phospholipid and protein.

2. Expressed on a protein basis the mitochondria have relatively low phospholipid and RNA contents, whereas the acid-soluble phosphate is higher than that of the microsomes. These facts, and particularly the very high RNA content of the microsomes, point to a general similarity to corresponding particles of animal origin.

3. During the first 4 days of germination there is an increase in mitochondrial protein and this is accompanied by an increase in all of the other components. After the fifth day these components decrease, especially the acid-soluble phosphate. The proportion of RNA and phospholipid to protein nitrogen remains more or less constant throughout, but that for acid-soluble phosphate declines.

4. A similar decline in acid-soluble phosphate is noted in the microsomes; in these particles there is no increase in protein or other components in the first few days of germination.

5. The water content of the mitochondria has been determined and its variation in response to media of different tonicity has been measured.

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Mitochondria in the Endosperm of the Germinating Castor Bean: a Developmental Study

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The fatty endosperm tissue of the germinating castor-bean seedling in the space of a few days progresses from the resting condition in the dry seed, through a phase of increasing metabolic activity and growth, to one of decreasing activity and depletion of its substance as its reserves are transported to the growing seedling attached to it. In 8–9 days the tissue has thus completed a cycle of activation, growth and ageing which in many other plant tissues occupies a period of several weeks or months. It was therefore excellent material in which to study the changes that occur in the mitochondria of a developing and ageing tissue. To this end preparations have been made at daily intervals for the determination of such quantities as: mitochondrial content, microsomal content, the intrinsic oxidative activity of the mitochondria under optimum conditions, the efficiency of oxidative phosphorylation and sensitivity to the uncoupling agent dinitrophenol. A preliminary account of this work has appeared elsewhere (Akazawa & Beevers, 1956).

MATERIALS AND METHODS

Most of these have been described in the previous paper (Akazawa & Beevers, 1957). All of the mitochondrial experiments were carried out with the crude unfractionated suspensions which, as before, were prepared so that a known number of beans and weight of endosperm tissue was represented by the volume (1 ml.) of suspension used in each instance.

In experiments in which oxidative phosphorylation was measured, hexokinase (Sigma, Chemical Co., St Louis, Mo., U.S.A.; 1 mg./2 ml. of reaction mixture), glucose (0·05 M) and sodium fluoride (0·02 M) were present in addition to the full complement of cofactors described by Beevers & Walker (1956). An oxidation period of 30 min. was allowed and the reaction was terminated by transferring a 1 ml. volume to 5 ml. of cold M-$HClO_4$. Phosphate was determined according to Fiske & Subbarow (1925).

Adenosine triphosphatase activity determinations. Mitochondrial suspension or supernatant (1 ml.) was added to 5 mg. of adenosine triphosphate (sodium salt, Pabst Brewing Co., Milwaukee, U.S.A.; ATP) and incubated at 25° for 30 min. The liberation of inorganic phosphate was used as the measure of activity.

RESULTS

Changes in mitochondrial content with age of seedling

During the first 5 days of germination there is a progressive increase in the bulk of mitochondria, as judged by the size of the pellet obtained after centrifuging. The data in Fig. 1 show that there is, in fact, a striking and regular increase in mitochondrial N during this time. After reaching a level about four times that at the 2 days stage, there is a fall in the amount of mitochondrial N/bean.

These changes made it of interest to find whether similar ones occurred in the microsomal particles. The microsomal N remained almost unchanged during the first 5 days (Fig. 2); after 5 days there is a decline in microsomal N which occurs concomitantly with that in mitochondrial N. The results in

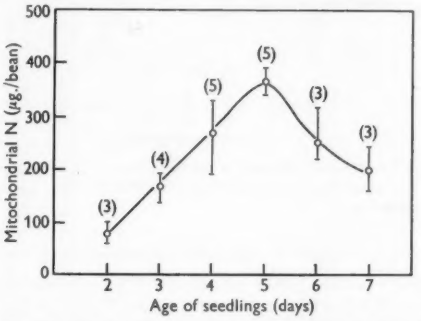

Fig. 1. Changes in the amount of endosperm mitochondrial N with age of seedling. (In Figs. 1, 3 and 4 the open points are the average values obtained in the stated number of experiments, and the vertical bar shows the extent of the variation encountered.)

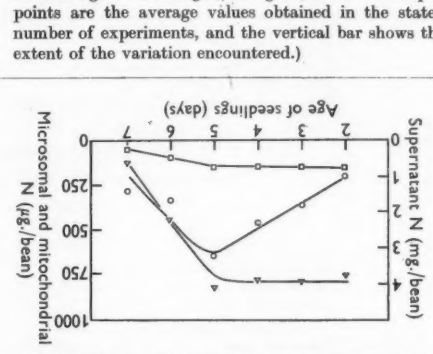

Fig. 2. Effect of age of the seedling on the amounts of total N contained in the supernatant (△), mitochondrial (○) and microsomal (□) fractions.

Fig. 2 also include values for the nitrogen content of the supernatant from the first centrifuging. In this series of experiments, determinations of the N content of mitochondria were made on unwashed sediments. It will be seen that even at its maximum, the percentage of N included in the two particulate fractions is less than 15 % of the total of the homogenate.

Changes in the q_{O_2} (μl. of O_2 absorbed/mg. of N/hr.) values with age of seedlings from which the mitochondria were extracted are shown in Fig. 3. The intrinsic activity of the mitochondria rises to a sharp maximum at 5 days and declines equally sharply thereafter.

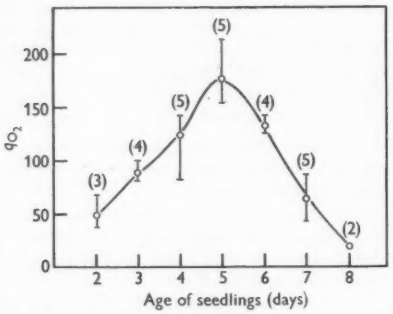

Fig. 3. Changes, with age of seedling, in the q_{O_2} values of the extracted mitochondria. Substrate was α-oxoglutarate.

Oxidative phosphorylation

Oxidative phosphorylation with a reasonably high efficiency occurs in mitochondria from 5 days seedlings which have, in addition to the usual complement of cofactors, the conventional hexokinase–glucose trapping system and fluoride (Table 1). The omission of either the trapping system or fluoride, or the addition of 2:4-dinitrophenol (DNP), leads to reductions in P/O (micromoles of phosphate esterified/microatoms of O_2 uptake) values. The addition of either hexokinase and glucose or DNP stimulates the O_2 uptake of the mitochondria. In subsequent experiments on oxidative phosphorylation the trapping system and fluoride were always added.

Table 2 shows the results of P/O determinations with a variety of substrates. It will be seen that in each case the oxidation is closely coupled to phosphorylation and satisfactorily high P/O values are obtained.

The changes which occur in phosphorylating efficiency as the seedlings age are shown in Fig. 4. The youngest seedlings provide mitochondria in which oxidation is tightly coupled with phosphorylation, for P/O values above 3 have been observed. Values considerably greater than 2 are obtained until the fifth day, after which there is an abrupt fall.

The effect of concentration of DNP on the O_2 uptake of mitochondria from 5-day-old beans is shown in Fig. 5. The greatest stimulation was observed at DNP concentrations of $1–2 \times 10^{-4}$ M. By using these concentrations, the changes in sensi-

Table 1. *Oxidative phosphorylation by 5 days mitochondria under different conditions*

The complete system contained 0·01 M-α-oxoglutarate, 0·05 M-glucose, 0·02 M-NaF, 1 mg. of adenosine triphosphate, 1 mg. of DPN, 0·5 mg. of cocarboxylase, 0·1 mg. of CoA, 1 mg. of hexokinase and 1 ml. of mitochondrial suspension in a volume of 2 ml.

	O_2 uptake (μatoms/30 min.)	Phosphate uptake (μmoles/30 min.)	P/O
Complete system	11·2	24·5	2·19
Without NaF	13·7	21·6	1·58
Without NaF and hexokinase	6·9	5·1	0·74
With DNP (2×10^{-4} M)	10·0	5·9	0·59
Without NaF and hexokinase; with DNP	17·4	0	0

Table 2. *Oxidative phosphorylation in the presence of different substrates*

Each system contained the complete additions shown in Table 1.

Substrate	Age of beans (days)	O_2 uptake (μatoms/30 min.)	Phosphate uptake (μmoles/30 min.)	P/O
α-Oxoglutaric acid	5	9·5	19·4	2·04
Malic acid	5	8·5	16·8	1·98
Citric acid	5	10·4	22·4	2·15
α-Oxoglutaric acid	3	5·9	12·3	2·1
Succinic acid	3	3·5	5·5	1·57
α-Oxoglutaric acid	5	10·5	20·8	1·98
Malic acid	5	11·0	22·0	2·0
Glutamic acid	5	12·8	25·6	2·0

tivity to DNP, which can be taken as a measure of the tightness of coupling, were examined, with the results shown in Fig. 6. The O_2 uptake of mitochondria from all seedlings younger than 5 days is greatly stimulated by DNP, but after this the response is smaller and has disappeared in 7-day-old seedlings.

One of the striking features of mitochondria from older endosperm is their high adenosine triphosphatase activity. The relationship between the development of this activity and the age of the seedlings is shown in Fig. 7. Adenosine triphosphatase activity is initially fairly low in the mitochondria, but a sharp increase begins after 5 days of germination; the activity of the supernatant, on the other hand, remains at a low level throughout.

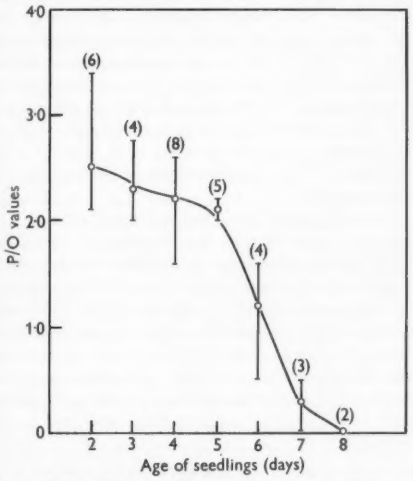

Fig. 4. Effect of age of the seedling on the efficiency of oxidative phosphorylation of the extracted mitochondria. Substrate was α-oxoglutarate.

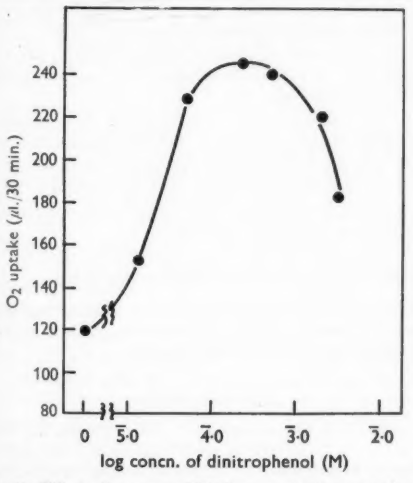

Fig. 5. Effect of a range of DNP concentrations on the O_2 uptake of mitochondria from 5-day-old beans. Substrate was α-oxoglutarate.

DISCUSSION

The results show clearly that there are striking differences in the amounts of mitochondrial nitrogen extractable by the present methods from beans of different ages. Further, in two important attributes, namely their intrinsic oxidative activity and their phosphorylating efficiency, the mitochondria show equally remarkable changes with age. If, as seems likely, these changes in activity reflect the properties of the native mitochondria *in vivo*, they are clearly of importance and must be considered, along with the differences in mitochondrial content, in attempts to gain an insight into the metabolism of the tissue itself.

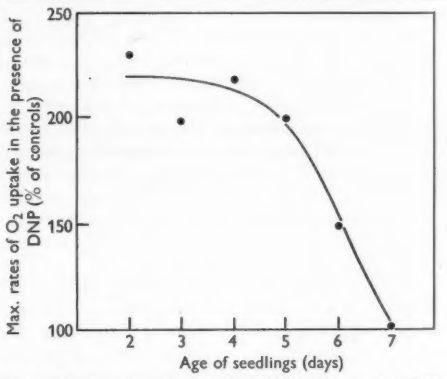

Fig. 6. Changes in the mitochondrial response to DNP during ageing of the seedling. (DNP, $1–2 \times 10^{-4}$ M.)

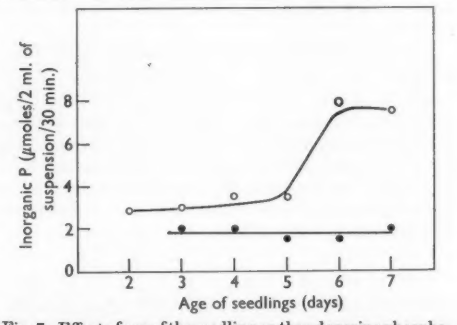

Fig. 7. Effect of age of the seedling on the adenosine phosphatase activities of mitochondria (○) and supernatant (●).

The production of new mitochondrial material during the early stages of germination increases the potential oxidative activity of the endosperm, and this production coincides with the increases in oxygen uptake actually observed in the intact tissue (Beevers & Walker, 1956). During this period of increasing mitochondrial nitrogen there is no decrease in the level of microsomal nitrogen. There is no evidence then in the castor-bean endosperm for the reciprocal changes in these constituents which have been described recently by Lund, Hanson & Kahn (1956) in growing corn roots.

During the first 5 days, as germination proceeds, not only is there an increase in the mitochondrial content, but the mitochondria themselves have higher intrinsic activity. The results of analyses for various components (Akazawa & Beevers, 1957) give no clue as to what might be responsible for the increasing q_{O_2} values of mitochondria, since the relative amounts of the components did not alter noticeably. Whatever the nature of this 'activation' it is clearly not accomplished at the expense of phosphorylating efficiency, because the P/O values are quite high.

This phase is succeeded by one of decreasing mitochondrial nitrogen and lowered q_{O_2} values as the endosperm ages, softens and loses its substance to the seedling proper. However, it appears that the potential usefulness of the mitochondria to the metabolism of the endosperm during this period is not limited solely by their decreasing amount and reduced q_{O_2} values. From the fifth day onwards there is a very sharp decline in the phosphorylating efficiency, as shown by the fall in the P/O value; by 7 days this ratio is very low indeed.

These changes in phosphorylating ability are reflected in the degree of stimulation of oxygen uptake by DNP. With mitochondria from the younger seedlings, the oxygen uptake is more than double the control rates, but subsequently the response decreases and none is observed in 7 days mitochondria. This seems to be the first report of stimulation of oxygen uptake of plant mitochondria by DNP.

It will be recalled (Akazawa & Beevers, 1957) that whereas the protein, phospholipid and RNA contents of the mitochondria declined together after the 5 days stage, that of the acid-soluble phosphate ('nucleotide') fraction showed a much steeper decline, which began somewhat earlier. It is now suggested that this change might be the underlying cause of the subsequent decline in oxidative and phosphorylative ability, and that the decrease in acid-soluble phosphate presages the early demise of the mitochondria. It is, of course, quite conceivable that at the 4 days stage some physical change occurs in the mitochondria which renders them 'leaky' and so instigates the progressive loss in acid-soluble phosphate. A further possibility, which might be difficult to separate from this, is that there is a breakdown of the components of the acid-soluble-phosphate fraction within the mitochondria themselves. It is significant in this regard that the adenosine triphosphatase of the mitochondria, although initially low, shows a striking rise after 5 days; but again, while this must certainly be of significance in limiting the oxidative, and more particularly the phosphorylative, abilities of the older native mitochondria, it may conceivably merely reflect their deteriorating physical condition. In this connexion it should be pointed out that it is not the increase in adenosine triphosphatase *per se* which is responsible for the lower P/O values at the later stages, since the enzyme was shown to be inhibited at the fluoride levels used during the P/O determinations.

The conclusions are that whereas in the first 5 days of germination the increasing metabolic activity of the endosperm is paralleled by changes in the content of mitochondria, which have a high respiration and phosphorylating ability, the subsequent declines in mitochondrial content and, more particularly, in q_{O_2} and P/O values, are such as to lower progressively any useful contribution of the mitochondria to the metabolism of the endosperm, which is, of course, approaching the end of its existence.

SUMMARY

1. Mitochondria prepared daily from the endosperms of germinating castor beans have been used to study changes in: (*a*) the amount of mitochondrial nitrogen/bean; (*b*) the q_{O_2} with α-oxoglutarate as substrate; (*c*) the phosphorylating efficiency (P/O ratios with α-oxoglutarate as substrate); (*d*) the sensitivity to 2:4-dinitrophenol; (*e*) the adenosine triphosphatase activity.

2. During the first 5 days there are striking increases in (*a*) and (*b*), the P/O values are considerably greater than 2, and the addition of dinitrophenol more than doubles the rate of oxygen uptake.

3. After 3 days there are marked decreases in (*a*), (*b*), (*c*) and (*d*) and an increase in (*e*).

4. The bearing of these changes on the metabolism of the endosperm tissue is discussed.

This work was financed by a grant to one of us (H.B.) from the National Science Foundation, and we wish to record our appreciation of this support.

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Side Reactions in the Deoxygenation of Dilute Oxyhaemoglobin Solutions by Sodium Dithionite

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Sodium dithionite ($Na_2S_2O_4$) is the commonest and most convenient reagent for the deoxygenation and reduction of haem pigments. The danger of undesirable side reactions between haem pigments and $Na_2S_2O_4$ or its oxidation products has been remarked by several workers (Hartridge & Roughton, 1923; Keilin, 1926, 1927; Zeile, 1932; Lemberg, Legge & Lockwood, 1941; Lemberg & Legge, 1949), and is particularly important in connexion with accurate kinetic studies of the deoxygenation of oxyhaemoglobin (HbO_2) in an excess of $Na_2S_2O_4$. Hartridge & Roughton (1923) studied the deoxygenation of HbO_2 by $Na_2S_2O_4$ in their rapid-flow apparatus, and concluded that the $Na_2S_2O_4$ did not react directly with the HbO_2, but simply removed the oxygen from the solution as fast as it was liberated, and that the direct and quantitative conversion of HbO_2 was by a simple first-order dissociation. These conclusions seemed to be confirmed by the more accurate and comprehensive measurements of Millikan (1933, 1936). Recently, however, evidence of a more complicated state of affairs was obtained by Legge & Roughton (1950), both in a re-inspection of Millikan's results and in new experiments on sheep HbO_2 by a similar method. First, it appeared that the first-order velocity coefficient increased significantly during the first third of the reaction, as was indeed to be expected on the basis of the intermediate-compound theory of haemoglobin (Hb) reactions (Roughton, 1949). Secondly, in some experiments at neutral pH the last stages of the deoxygenation seemed to be extremely slow, and to be associated, at least in part, with the formation of pigments in side reactions brought about by $Na_2S_2O_4$ oxidation products, which would invalidate the colorimetric method of analysis. The matter was not fully worked out, but it was suggested that a precursor of choleglobin might be the interfering substance. An initial increase of the first-order velocity coefficient has also been recorded for the deoxygenation of human HbO_2, but no slow terminal stage was observed (Dalziel, 1954). During incidental measurements, however, it was also noted that a perfectly stable Hb spectrum was not at once obtained on the addition of excess of $Na_2S_2O_4$ to a dilute, slightly alkaline HbO_2 solution in the absence of air, and that similar slow spectral changes in the absorption spectrum of a dilute Hb–$Na_2S_2O_4$ solution were induced by brief exposure to air. These observations were consistent with the conclusions of Legge & Roughton (1950) that oxidation products of $Na_2S_2O_4$ could bring about side reactions with the pigment.

The purpose of the experiments described here was to obtain evidence of the nature of these side reactions by a closer examination of the slow spectral changes which followed the deoxygenation of HbO_2 by $Na_2S_2O_4$, and the action of oxygen on a Hb–$Na_2S_2O_4$ solution, under various conditions of concentration and pH.

EXPERIMENTAL

Measurements of light absorption were made with a Beckman quartz photoelectric spectrophotometer (model DU) and are generally recorded as the extinction

$$(E = \log I_0/I).$$

Spectrokinetic measurements

In most of the experiments solid $Na_2S_2O_4$ was weighed into a dry 1 cm. optical cell, 3 ml. of buffered HbO_2 solution was quickly pipetted in and a layer of liquid paraffin at once added. The mixture was stirred under the paraffin layer with a fine glass rod, and more paraffin was added to fill the cell, which was sealed with a cover slip. In some experiments, 1·5 ml. of buffered $Na_2S_2O_4$ solution (stored under paraffin) was pipetted under the surface of 1·5 ml. of buffered HbO_2 solutions in a cell; in others, contact with air was completely eliminated by adding solid $Na_2S_2O_4$ in a sealed-glass capillary, which was broken under the surface of the HbO_2 solution and its protective layer of paraffin. Qualitatively similar results were obtained by all these methods. The time course of the extinction of the mixture was followed by making measurements at two or three wavelengths in rapid succession, or sometimes at a single wavelength, from about 1 min. after mixing until constant values were obtained. Similar measurements were made at other wavelengths on fresh preparations. The action of O_2 was studied on the same preparations; after a stable spectrum had been obtained, some of the paraffin was removed, and O_2 was bubbled through the solution from a capillary tube at 2–3 bubbles/sec. for 30 sec. The cell was then sealed and the extinction measured again from about 30 sec. later.

Control experiments, under all the test conditions, showed that the reaction between $Na_2S_2O_4$ and O_2 alone did not

cause significant changes of extinction in the region 400–650 mμ, and there were no visible signs of turbidity or denaturation in the experiments. The changes of pH, due to acidic oxidation products of the $Na_2S_2O_4$, were small in the buffered solutions.

Reagents and derivatives

Haemoglobin. The HbO_2 solutions were haemolysates of washed human red cells; saponin was used to give complete haemolysis. The Hb concentrations were estimated from extinction measurements and the extinction coefficients of HbO_2 (Lemberg & Legge, 1949).

Sodium dithionite. Ordinary commercial samples were used.

Hydrogen peroxide. A.R. '20 vol.' reagent was diluted in buffer solution as required. The stock solution was repeatedly analysed by permanganate titration.

Buffer solutions. These were Clarke and Lubs buffer 0·05 M-KH_2PO_4–NaOH (pH 6·0–8·0) and 0·05 M-H_3BO_3–KCl–NaOH (pH 8·0–10·0) (Vogel, 1939).

Reactant concentrations. Initial concentrations in the reaction mixtures are referred to throughout. Hb concentrations are expressed in m-equiv./l.; the expression mEq.-Hb is equivalent to mM-haem, and m-equiv. extinction coefficients. $\epsilon_{mEq.}$, refer to these concentration units (Dalziel & O'Brien, 1954).

RESULTS

Side reactions in the deoxygenation of oxyhaemoglobin by dithionite

The curves of Fig. 1 built up from duplicate or triplicate measurements on two series of mixtures represent the spectral absorption of mixtures of HbO_2 and $Na_2S_2O_4$ recorded 1 and 15 min. or more after mixing. The spectral absorption 1 min. after mixing is a typical Hb curve, $\lambda_{max.}$ 430, 555 mμ, but during the next few minutes the intensity of absorption increased over both the Hb maxima (420–445 and 540–575 mμ), and decreased over the saddle, beyond 585 mμ, and also in the region 400–415 mμ. The crossing points of the curves at which the intensity remained constant are at about 417, 448, 536 and 579 mμ. Spectrokinetic curves for four wavelengths, obtained with two HbO_2 concentrations, pH 8·5, are shown in Fig. 2. Similar results were obtained in experiments at pH 5·9 and 9·5; in the acid solutions the slow changes were distinctly smaller in magnitude and shorter in duration.

The effects of variation of the reactant concentrations and the pH on the magnitude of the slow increase of extinction at 430 mμ were systematically studied (Fig. 3). The variation of the final stable extinction coefficient with the conditions shows that HbO_2 is lost in the overall reaction as products other than Hb. Both the 1 min. and the final, 20 min., values of the extinction coefficient become greater with (i) fall of pH, (ii) increase of $Na_2S_2O_4$ concentration and (iii) increase of pigment concentration. Thus the percentage yield of Hb is smallest with dilute HbO_2 solutions, small $Na_2S_2O_4$ concentrations and high pH, and under these conditions the slow increase of extinction at the Hb maximum is most marked.

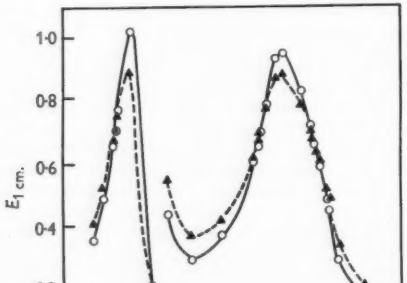

Fig. 1. Spectral absorption of solutions of HbO_2 to which 0·1 g. of $Na_2S_2O_4$/100 ml. has been added, recorded 1 min. (▲) and 15 min. (○) after addition of $Na_2S_2O_4$. Concentration of HbO_2 solutions for measurements between 400 and 450 mμ; 8×10^{-3} mEq.; between 450 and 650 mμ, 8×10^{-2} mEq.

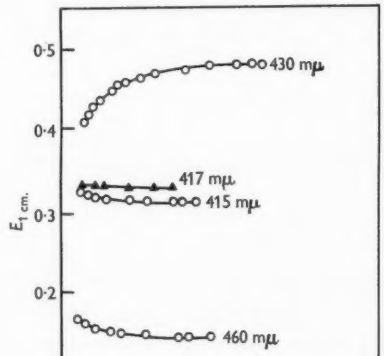

Fig. 2. Changes of extinction with time at four wavelengths 1 min. after addition of 0·2 g. of $Na_2S_2O_4$/100 ml., to HbO_2 solutions of pH 8·5. HbO_2 concentrations: 4×10^{-2} mEq. for 415, 417, and 430 mμ, and 3×10^{-2} mEq. for 460 mμ.

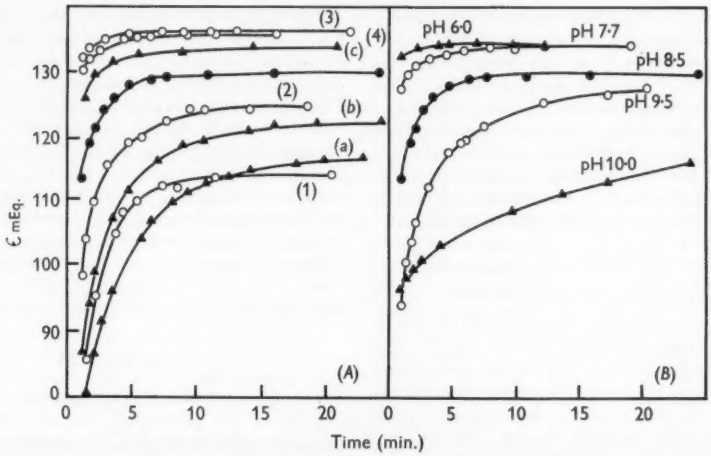

Fig. 3. Time course of the extinction coefficient at 430 mμ from 1 min. after the addition of $Na_2S_2O_4$ to HbO_2, with various reactant concentration and pH values. (*A*) pH 8·5 and various reactant concentrations: ●, 0·2% $Na_2S_2O_4$ and 5×10^{-3} mEq.-HbO_2; ○, 0·2% $Na_2S_2O_4$ and HbO_2 concentrations (1) 1·25, (2) 2·5, (3) 10, (4) 20×10^{-3} mEq.; ▲, 5×10^{-3} mEq.-HbO_2, and $Na_2S_2O_4$ concentrations (g./100 ml.): (*a*) 0·05, (*b*) 0·1, (*c*) 0·4. (*B*) 0·2% $Na_2S_2O_4$ and 5×10^{-3} mEq.-HbO_2 at various pH values.

No evidence of side reactions had been noted in kinetic studies of the deoxygenation in a rapid-reaction apparatus (Dalziel, 1954), when relatively high pigment concentrations had been used. In accordance with the results of earlier workers, the deoxygenation had apparently been complete in less than a second; when the flow was stopped the extinction of the products had immediately assumed a value close to that of a calibration fluid consisting of fully reacted Hb and had then remained unchanged for at least a minute. More careful and prolonged observation, however, showed that slow changes of the kind described did in fact occur; the extinction of the products increased slightly at 430 mμ and decreased very slightly at 460 mμ during a period of 5 min. Confirmation of these small changes was sought by stopped-flow measurements in an attachment to the observation tube of optical-path length 1 cm., which permitted accurate measurements on more dilute pigment solutions. Buffered solutions of $Na_2S_2O_4$ and HbO_2 at pH 8 were mixed by flow, and when steady conditions were attained the flow was suddenly stopped and the extinction of the mixture was recorded against time. During flow through the observation tube the extinction at 430 mμ increased rapidly as Hb was formed, but immediately the flow was stopped it began to decrease and continued to fall for about 30 sec., and then increased slowly until a constant value was attained after about 10 min. (Fig. 4). The reality of these small changes was established by control experiments; when a Hb–$Na_2S_2O_4$ solution

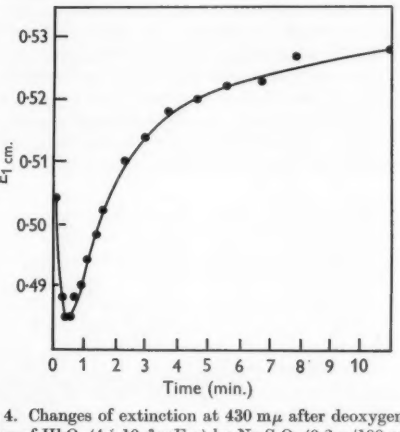

Fig. 4. Changes of extinction at 430 mμ after deoxygenation of HbO_2 (4×10^{-3} mEq.) by $Na_2S_2O_4$ (0·2 g./100 ml.) at pH 8·0, recorded in the rapid-reaction apparatus by the stopped-flow method.

was substituted for the HbO_2 solution, a perfectly constant value of the extinction was recorded under similar conditions. The results suggest that two reactions follow the rapid deoxygenation.

The addition of $Na_2S_2O_4$ to dilute methaemoglobin solutions was followed by similar slow spectral changes. When red-cell suspensions were deoxygenated by $Na_2S_2O_4$, however, a practically stable spectrum was obtained at once. It seemed

therefore that the side reactions were not directly connected with the deoxygenation of HbO_2, but arose from the action of an oxidation product of $Na_2S_2O_4$ on Hb (cf. Legge & Roughton, 1950). This conclusion was strengthened by the observation that exposure of a $Na_2S_2O_4$ solution to air immediately before mixing with HbO_2 enhanced the side reactions. The effect was not permanent, and disappeared when the $Na_2S_2O_4$ solution was allowed to stand in the absence of air for a few minutes.

Spectral changes induced by oxygen in haemoglobin–dithionite solutions

After the slow spectral changes which follow deoxygenation had been recorded in the experiments described previously (Figs. 1, 2), O_2 was passed into the products for 30 sec. Measurements showed that during the action of O_2 the intensity of absorption had fallen at both the Hb maxima, and risen at the minimum and beyond 585 mμ, and during the next few minutes the original spectrum was largely restored by changes in the reverse direction at most wavelengths. These slow changes appeared to be identical throughout the spectrum in magnitude, direction and rate with those which had followed the initial deoxygenation (Fig. 1). When a stable spectrum was again obtained, it differed from the initial Hb spectrum (Fig. 1) only in having smaller maxima and greater absorption in the red. Some Hb had therefore been destroyed in the overall reaction. No distinct spectral changes were associated with this destruction except perhaps a suggestion of a small maximum near 630 mμ.

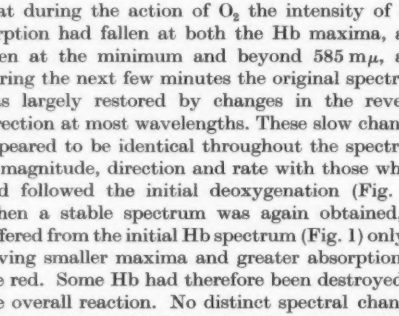

Fig. 5. Effect of oxygen on the extinction of haemoglobin–dithionite solutions. HbO_2 and $Na_2S_2O_4$ were mixed at zero time (see Fig. 2) and oxygen was passed for 20 sec. at times indicated by arrows. Concentrations of reactants are given in Fig. 2.

Spectrokinetic curves of the slow changes following deoxygenation (see also Fig. 2) and the subsequent action of O_2 are given in Fig. 5, and bring out the similarity between the two reactions. The curve for 430 mμ is representative of the changes at both the Hb maxima; the considerable decrease of absorption which occurred during the passage of O_2 was largely but not completely reversed in the subsequent slow reaction. A reverse sequence of smaller changes occurred at 460 mμ and at other wavelengths near the minimum and beyond 580 mμ. From 400 to 417 mμ, both the immediate action of O_2 and the subsequent slow reaction were accompanied by small decrease of extinction.

DISCUSSION

It has been known for some time that the action of oxygen on Hb in $Na_2S_2O_4$ solution results in the formation of choleglobin-like oxidation products, manifest by increased light absorption in the red and decreased absorption at shorter wavelengths, notably in the Soret region (Lemberg *et al.* 1941; Lemberg & Legge, 1949). The present experiments show that an unstable derivative of Hb is also formed under conditions which favour the formation of an oxidation product of $Na_2S_2O_4$, and is slowly reconverted, in part, into Hb in the presence of excess of $Na_2S_2O_4$. These reactions also accompany the conversion of HbO_2 and methaemoglobin into Hb by $Na_2S_2O_4$ in dilute solution. To account for the observed spectral changes, the unstable derivative, unlike the stable degradation products, must have (*a*) absorption in the Soret region about equal to, or slightly greater than, that of Hb at 400–420 mμ and considerably less than that of Hb around 430 mμ; (*b*) a greater absorption in the regions 450–530 mμ and 580–650 mμ; (*c*) isosbestic points with Hb at about 417, 448, 536 and 579 mμ (Fig. 1). Neither HbO_2 nor methaemoglobin satisfies these conditions.

Both the formation of the unstable derivative and the irreversible degradation of the Hb were favoured by low Hb and $Na_2S_2O_4$ concentrations and by slightly alkaline conditions, consistent with the view that the unstable compound is an intermediate in the degradation. The effect of $Na_2S_2O_4$ concentration also suggests the origin of the reactions. If $Na_2S_2O_4$ itself were involved, it would be expected that the extent of the reactions would either increase with the $Na_2S_2O_4$ concentrations or be independent of it. If a stable oxidation product of $Na_2S_2O_4$ were involved, the extent of the reactions should also be independent of the $Na_2S_2O_4$ concentration, with constant total oxygen concentration. The observed inhibitory effect of $Na_2S_2O_4$ agrees with the assumption that an intermediate product of the reaction of $Na_2S_2O_4$ with oxygen, which is ultimately destroyed by the excess of $Na_2S_2O_4$, is

responsible for these reactions. This would also explain why the activation brought about by exposure of the $Na_2S_2O_4$ solution to air is only temporary and disappears when the $Na_2S_2O_4$ is allowed to stand in absence of air.

An unstable oxidation product of $Na_2S_2O_4$ is hydrogen peroxide (Jellinek, 1912; Conant & Scott, 1926), which reacts with several haem pigments, including methaemoglobin, to form unstable compounds with well-defined spectral characteristics, and with Hb to form choleglobin (Lemberg *et al.* 1941). An unstable Hb–peroxide compound was postulated by Lemberg *et al.* (1941) as the precursor of choleglobin. It has been shown (Dalziel & O'Brien, 1957) that the unstable compound detected and characterized spectrophotometrically in the experiments described here is also formed on the addition of hydrogen peroxide to Hb in $Na_2S_2O_4$ solution.

The results underline the dangers of the use of $Na_2S_2O_4$ in quantitative experiments with Hb derivatives in the presence of oxygen. The losses involved in the conversion of HbO_2 into Hb by $Na_2S_2O_4$ in dilute, slightly alkaline solution may be of a size that may produce considerable errors in spectrophotometric measurements made during the life of the unstable intermediate. Advantage may sometimes be taken of the stability of the carbon monoxide derivatives to avoid such errors (cf. Lemberg *et al.* 1941); if HbO_2 is reduced by $Na_2S_2O_4$ and then converted into carboxyhaemoglobin the absorption spectrum of the product shows greater absorption in the red than that of a similar preparation in which the HbO_2 solution was first saturated with carbon monoxide and then treated with $Na_2S_2O_4$. It is to be expected that considerable irreversible degradation may accompany the reconversion of Hb into HbO_2 by oxygenation of $Na_2S_2O_4$ solution. For sheep HbO_2 prepared in this way Beznak (1948) found $\lambda_{max.} = 412\ m\mu$, $\epsilon_{mEq.} = 72$, which may be compared with the generally accepted values of $\lambda_{max.} = 415\ m\mu$, $\epsilon_{mEq.} = 128$ (Lemberg & Legge, 1949), and for sheep myoglobin, similarly prepared $\lambda_{max.} = 410\ m\mu$, $\epsilon_{mEq.} = 130$. In contrast, other pairs of analogous Hb and myoglobin derivatives gave extinction coefficients in the Soret region in close agreement with one another. Beznak devised a spectrophotometric method for the analysis of mixtures of Hb and myoglobin on the basis of the decrease of extinction in the Soret region which accompanied reoxygenation of $Na_2S_2O_4$ solutions. The method probably depends, not upon any real difference between the molecular extinction coefficients of HbO_2 and oxymyoglobin, but upon a greater susceptibility of Hb to oxidative degradation, during the oxygenation, to choleglobin and other products with little specific absorption in the Soret region. There is direct evidence that myoglobin is more resistant than Hb to degradation by hydrogen peroxide in the presence of $Na_2S_2O_4$ (Dalziel & O'Brien, 1957).

Sodium dithionite is evidently an unsatisfactory reagent for accurate kinetic studies of the deoxygenation of HbO_2, but no suitable alternative has yet been found. The conclusion of Legge & Roughton (1950) that the occasional slow terminal stage recorded by colorimetric analysis was an artifact is substantiated by the present work, but the fact that the increasing first-order velocity coefficient in the early stages of the reaction is found in spectrokinetic measurements at both 415 and 430 $m\mu$ (Dalziel, 1954) makes it likely that this is a true kinetic feature of the deoxygenation. It must be added, however, that discrepancies have been discovered between the rate at which carbon monoxide displaces oxygen from combination with Hb and the rate of dissociation of HbO_2 in the presence of $Na_2S_2O_4$, which may indicate that $Na_2S_2O_4$ has a direct action on the pigment and modifies the kinetics of the dissociation, at least in neutral solution (Gibson & Roughton, 1955; Gibson, 1955).

SUMMARY

1. The addition of an excess of sodium dithionite to dilute solutions of oxyhaemoglobin or methaemoglobin does not give a quantitative yield of haemoglobin. Side reactions occur in which part of haemoglobin is degraded to products with relatively low specific absorption. Similar reactions are induced by the brief oxygenation of dilute haemoglobin solutions containing an excess of sodium dithionite.

2. Evidence has been obtained that these reactions are brought about by an oxidation product of sodium dithionite, and involve the temporary formation of an unstable intermediate compound, which may be degraded or slowly reconverted into haemoglobin.

3. The significance of these results in relation to quantitative spectrophotometric and kinetic measurements with oxyhaemoglobin and haemoglobin is discussed.

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Spectrokinetic Studies of the Reaction of Hydrogen Peroxide with Haemoglobin in Dithionite Solutions

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It was shown (Dalziel & O'Brien, 1957) that the action of oxygen on haemoglobin in dithionite solution converts part of the haemoglobin into an unstable derivative which, with excess of dithionite, largely reverts to haemoglobin. Small amounts of stable, choleglobin-like products were formed. These reactions were attributed to the action of hydrogen peroxide formed in the autoxidation of dithionite (cf. Lemberg, Legge & Lockwood, 1941*b*; Legge & Roughton, 1950).

In this paper studies of the reaction of hydrogen peroxide with haemoglobin in an excess of dithionite are described.

EXPERIMENTAL

Buffered solutions of H_2O_2 and of haemoglobin (Hb) containing $Na_2S_2O_4$ were studied in the rapid-reaction apparatus (Dalziel, 1953, 1954; Dalziel & O'Brien, 1954*a*). The solutions were mixed in the absence of air and the extinction was recorded 0·2 or 0·8 sec. after mixing. Stopped-flow measurements were also made. The extinction at zero time was obtained by flowing the Hb–dithionite reactant with a dithionite solution instead of H_2O_2. Spectrokinetic curves were drawn from such measurements at different wavelengths.

Samples of the reaction mixture, and control samples of the Hb reactant mixed with $Na_2S_2O_4$ solution instead of H_2O_2, were collected from the apparatus, covered with a layer of liquid paraffin and their absorption spectra subsequently measured.

Reagents and derivatives

Hydrogen peroxide. A.R. '20 vol.' reagent was diluted in buffer solution as required. The stock reagent was repeatedly analysed by titration with $KMnO_4$.

Haemoglobin. Except where otherwise stated, the experiments were made with Hb solutions free from catalase, prepared from haemolysates of washed human red cells by treatment with alumina. The Hb concentration was estimated spectrophotometrically after conversion into cyanmethaemoglobin (Drabkin & Austin, 1935–36; Dalziel & O'Brien, 1954*a*). Solid $Na_2S_2O_4$ (a fresh dry commercial sample stored in a sealed bottle) was put in the reactant vessel of the apparatus, and oxyhaemoglobin (HbO_2) solution poured in and immediately covered with paraffin.

Metmyoglobin (MetMb). Crystalline horse metmyoglobin (Theorell & Ehrenberg, 1951) was dissolved in water, dialysed free from $(NH_4)_2SO_4$, and finally dialysed against borate buffer (pH 8·5). The concentration of MetMb in the stock solution was determined spectrophotometrically after conversion into cyan-metmyoglobin (Drabkin, 1945).

Buffer solutions. These were Clarke and Lubs buffer 0·05 M-KH_2PO_4–NaOH (pH 6·0–8·0) and 0·05 M-H_3BO_3–KCl–NaOH (pH 8·0–9·5) (Vogel, 1939).

Denatured globin carboxyhaemochromogens. These were prepared by the method of Lemberg *et al.* (1941*b*) and will be referred to as CO-haemochromogens.

Reactant concentrations. Initial concentration in the reaction mixture is referred to throughout. Hb concentrations are expressed in m-equiv./l.; mEq.-Hb is equivalent to mM-haem, and m-equiv. extinction coefficients, $\epsilon_{mEq.}$, refer to these concentration units; the range of Hb concentrations used was 0·02–0·50 m-equiv./l. The concentration of $Na_2S_2O_4$ was varied from 0·05 to 2·0% (w/v). The H_2O_2 concentrations were in the range 0·5–20 m-moles/l.

RESULTS

Products of the overall reaction

The reaction between Hb in $Na_2S_2O_4$ solution and H_2O_2 was studied spectrophotometrically at pH 5·9, 7·0, 7·7, 8·0, 8·4, 9·0 and 9·5. A comparison of the absorption spectra at pH 5·9 and 7·7 with that of Hb in $Na_2S_2O_4$ solution illustrates the main features of the overall reaction (Fig. 1*A*). Depending upon the pH, there is a variable loss of Hb and the formation of substances absorbing in the orange part of the spectrum; these changes are confirmed by the spectra of the reaction products after addition of alkali and CO (Fig. 1*B*).

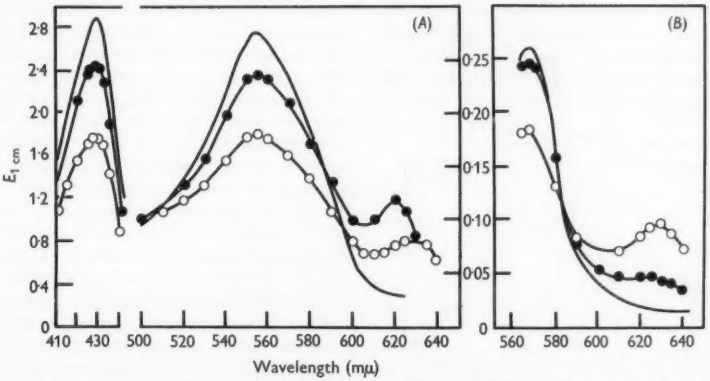


Fig. 1. (*A*) Absorption spectra of the products of the reaction of 0·02 mEq.-Hb, 0·1% $Na_2S_2O_4$ and 2 mM-H_2O_2 at pH 5·9 (●) and pH 7·7 (○). (*B*) Absorption spectra of CO-haemochromogen derivatives of the products of reaction of 0·02 mEq.-Hb, 0·1% $Na_2S_2O_4$ and 2 mM-H_2O_2 at pH 5·9 (●) and pH 7·7 (○). Curves without experimental points are for 0·02 mEq.-Hb in 0·1% $Na_2S_2O_4$ and CO-protohaemochromogen prepared therefrom.

At pH 5·9, where the destruction of Hb is small, there is a maximum at 620 mμ, which is largely destroyed upon the addition of alkali and CO. The only known pigment with properties consistent with these results is sulphaemoglobin (Lemberg & Legge, 1949).

At pH 7·7, and in more alkaline solutions, Hb is partly destroyed, and a band at 630 mμ appears. This band was unaffected by treatment with alkali and CO (Fig. 1*B*) or by CO alone. Addition of alkali alone, however, resulted in a shift of this band to 617 mμ. Of the known pigments, choleglobin has the properties which best fit these facts (Lemberg & Legge, 1949). The results at pH 7 were consistent with a mixture of choleglobin and sulphaemoglobin. In all reactions, the bulk of the Hb was apparently unchanged. The yield of choleglobin decreased with increase of pH above 8·4, although the extent of Hb and protohaem destruction remained fairly constant. Detailed study of the reaction of Hb with H_2O_2 in a reducing medium showed that it was a complex one and composed of a number of stages, which are described below.

Formation of intermediate compound

The time course of the changes in light absorption during the reaction at pH 8·4 from 0·2 sec. after mixing is shown in Figs. 2*A*, 2*B* and 3. The occurrence of simultaneous discontinuities in the spectrokinetic curves of Fig. 2 shows that at least three reactions occur. The first is a rapid one, complete in 5–10 sec., in which the spectrum of the mixture changes completely, and is succeeded by a slow decline of the extinction at all wavelengths. Finally, after about 60 sec., a third slow reaction begins which results in partial reversal of the initial changes at all wavelengths except those near 630 mμ. The significance of these changes is brought out in Fig. 3, which gives the integrated absorption spectra at intervals after mixing. At 20 sec. the spectrum of Hb has been replaced by a different one, with absorption maxima at 417, 545 and 582 mμ. This persists up to 60 sec. without significant change of shape, but with slowly decreasing intensity, and then slowly reverts to a Hb spectrum of reduced intensity with an additional maximum at 630 mμ, which may be attributed to choleglobin. This spectrum is referred to as the intermediate spectrum.

Similar spectrokinetic studies were made under various conditions: at pH 8·4 with reactant concentrations covering the ranges 0·02–0·1 mEq.-Hb, 0·05–2·0% $Na_2S_2O_4$ and 0·5–5·0 mM-H_2O_2; at pH 9–10 with up to 0·5 mEq.-Hb and 20 mM-H_2O_2 (cf. Dalziel & Ehrenberg, 1955); and at pH 5·9, 7·0 and 7·7 with 0·02 mEq.-Hb, 0·1% $Na_2S_2O_4$ and 2 mM-H_2O_2. In every experiment an excess of $Na_2S_2O_4$ over H_2O_2 was used, and the bulk of the Hb was apparently recovered unchanged. Spectrokinetic curves were recorded at the absorption maxima of Hb, choleglobin and the intermediate spectrum and in many cases at other wavelengths. The same sequence of extinction changes occurred in all the experiments at pH 7·7–10. The same intermediate spectrum was obtained, and the $\epsilon_{mEq.}$ values were reproducible: 90 ± 3 at 417 mμ, 50 ± 4 at 430 mμ, $9{\cdot}0 \pm 0{\cdot}4$ at 545 mμ, $9{\cdot}6 \pm 0{\cdot}4$ at 582 mμ. With increase of pH from 7·7 to 10: (i) the life of the intermediate spectrum increased markedly; (ii) the rate at which it declined during its life decreased; (iii) the rate at which it was finally displaced by an Hb spectrum decreased. At pH 7 similar results

were obtained, but the intermediate spectrum had a lifetime of only a few seconds. At pH 5·9, the extinction changes were in the same sense as but smaller than those at higher pH values, and there was no 'steady state' in which a characteristic spectrum persisted.

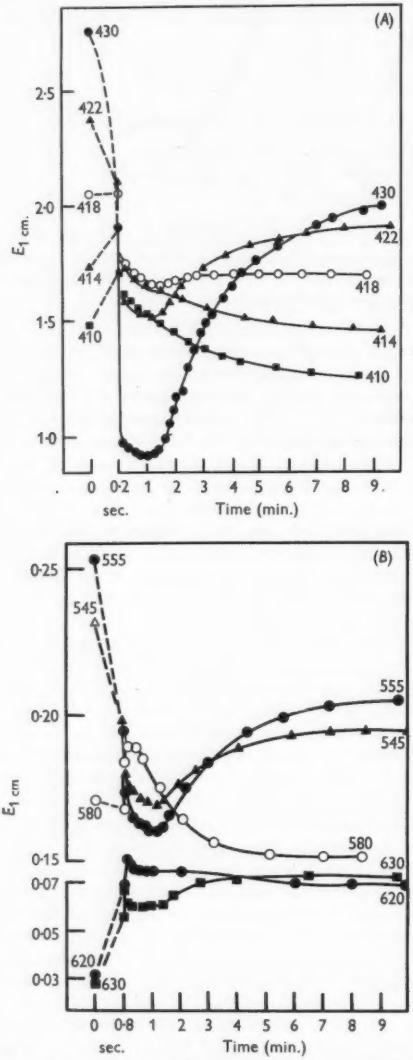


Fig. 2. (*A*) and (*B*) Plots of extinction against time after adding H_2O_2 to Hb and $Na_2S_2O_4$. Wavelengths shown on the curves are in mμ. The reaction mixture contained initially 0·02 mEq.-Hb, 0·1% $Na_2S_2O_4$ and mM-H_2O_2, at pH 8·4.

In these experiments the decrease of the extinction at 430 mμ to a reproducible level shows that Hb is completely displaced in the initial reaction. The symmetry, persistence and reproducibility of the maximum at 417 mμ under varied conditions justifies the conclusion that this intermediate spectrum is largely due to a single Hb derivative, some of which undergoes degradation to products with little specific absorption, but the bulk is slowly reconverted into Hb.

Side reaction accompanying formation of intermediate compound. There are, however, indications that more than one reaction occurs in the initial stage of the reaction. In Fig. 2*A*, the extinction at 410–416 mμ increases between 0 and 0·2 sec. and then decreases to the level of the intermediate spectrum at 2–3 sec. These changes can be understood by the following experiment. A flowing-reaction mixture (0·2 mEq.-Hb, 0·1% $Na_2S_2O_4$, 3 mM-H_2O_2, pH 8·4) was observed spectroscopically in the rapid-flow apparatus; 0·09 sec. after mixing the Hb–$Na_2S_2O_4$ and H_2O_2 solutions, the bands of HbO_2 ($\alpha = 579$ mμ; $\beta = 542$ mμ) were seen; from 0·3 to 0·5 sec., the single broad band of Hb was present but, on stopping the flow, the spectrum of the intermediate compound ($\alpha = 585$; $\beta = 545$ mμ) at once appeared.

The extinction changes in the Soret region 415 mμ ($\lambda_{max.}$ HbO_2), 430 mμ ($\lambda_{max.}$ Hb) and 421 mμ in the early stages of the reaction are shown in Fig. 4. Hb and HbO_2 have the same molar extinction at 421 mμ; Hb and the intermediate compound have about equal extinctions at 415 and 421 mμ, but at 430 mμ that of the transient compound is much smaller (cf. Figs. 2*A*, 3). Thus the results of Fig. 4

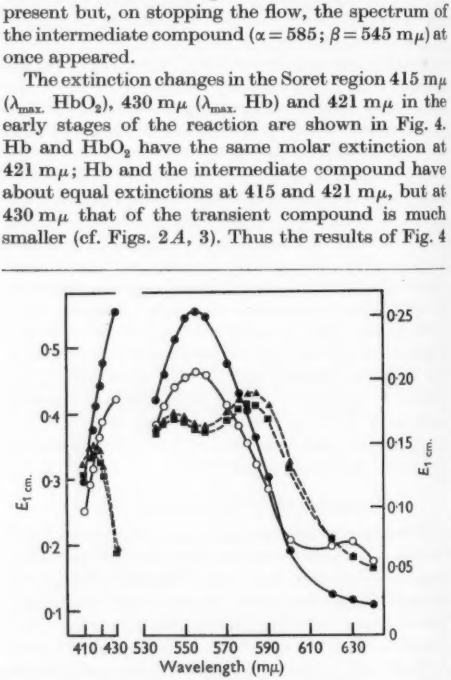


Fig. 3. Absorption spectrum of a reacting mixture of 0·02 mEq.-Hb, 0·1% $Na_2S_2O_4$ and mM-H_2O_2, pH 8·4, at intervals after adding the H_2O_2: ●, initial spectrum (Hb); ▲, 20 sec.; ■, 60 sec.; ○, 10 min.

are consistent with the sequence of reactions: (1) Hb → HbO_2, complete within 0·01 sec.; (2) HbO_2 → Hb, from 0·01 to 0·25 sec.; (3) Hb → transient compound, overlapping (2) and becoming apparent as a decrease of extinction at 430 mμ after 0·3 sec.

The overall decrease of $\epsilon_{mEq.}$ of the reaction mixture at 430 mμ from 0 to 0·2 sec. may be taken as a measure of the proportion of Hb converted into the intermediate compound in this time. The values in Table 1 for reaction mixtures of different composition suggest that at pH 8·4 this proportion increases with the H_2O_2 concentration, but is independent of the Hb or $Na_2S_2O_4$ concentrations.

Lifetime of the intermediate compound. The life of the compound [measured by the time taken for the extinction at 430 mμ to reach a minimum (cf. Fig. 2*A*)], varies directly with the H_2O_2 concentration and inversely with the $Na_2S_2O_4$ and pigment concentrations, and is shortened by catalase (Table 2).

The rate at which the intermediate spectrum declined during its lifetime was not affected by a fivefold change of H_2O_2 concentration at pH 8·4, but increased with the $Na_2S_2O_4$ and pigment concentrations.

Haemoglobin regeneration and choleglobin formation. Except near 630 mμ, the initial changes of spectral absorption are largely reversed in the final stages of the reaction. At 630 mμ the extinction increases during the formation of both the intermediate compound and Hb, showing that choleglobin accumulates. The effects of varying the reactant concentrations, at pH 8·4, are represented in Table 3, where the amounts of Hb destroyed are expressed in terms of the percentage decrease of extinctions, $100\Delta E/E$, at the Hb (or CO-haemochromogen) maxima, and the amounts of choleglobin formed in terms of the increase of light absorption of haemochromogen at 630 mμ. The data show that Hb destruction and choleglobin formation do not change in a parallel manner.

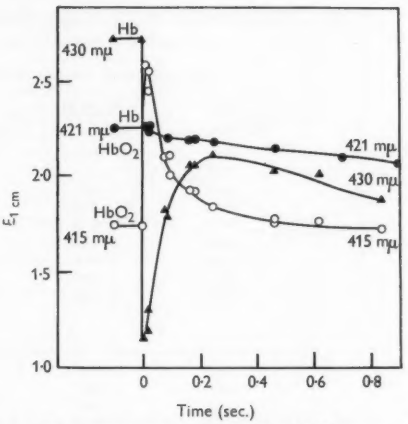


Fig. 4. Plots of extinction at 415, 421 and 430 mμ against time after the addition of H_2O_2 to Hb and $Na_2S_2O_4$: 0·02 mEq.-Hb, 0·1% $Na_2S_2O_4$, mM-H_2O_2, pH 8·4, showing the early stages of the reaction.

Table 1. *Extinction coefficients* ($\epsilon_{mEq.}$) *after the addition of hydrogen peroxide to haemoglobin–dithionite solutions*

Extinction coefficients ($\epsilon_{mEq.}$) were measured at 430 mμ, 0·2 sec. after the addition of hydrogen peroxide to haemoglobin–dithionite solutions, at pH 8·4. The rate of formation of the transient compound ($\epsilon_{mEq.}$ = 50) from haemoglobin ($\epsilon_{mEq.}$ = 135) with various reactant concentrations is shown.

Composition of reaction mixture			
Hb (m-equiv./l.)	$Na_2S_2O_4$ (g./100 ml.)	H_2O_2 (m-moles/l.)	$\epsilon_{mEq.}$
0·10	0·10	1·0	102
0·02	0·10	1·0	95
0·02	0·20	1·0	100
0·02	0·20	5·0	64
0·02	0·80	5·0	66

Table 2. *Effect of reactant concentrations and catalase on the life of the transient spectrum at* pH 8·4

Composition of reaction mixture				Life of transient spectrum (sec.)
Hb (m-equiv./l.)	$Na_2S_2O_4$ (g./100 ml.)	H_2O_2 (m-moles/l.)	Catalase	
0·02	0·1	0·5	Absent	28
0·02	0·1	1·0	Absent	60
0·02	0·1	2·0	Absent	160
0·02	0·2	1·0	Absent	30
0·02	0·2	5·0	Absent	111
0·02	0·8	5·0	Absent	33
0·02	0·05	1·0	Absent	210
0·02	0·8	1·0	Absent	15
0·05	0·1	1·0	Absent	24
0·10	0·1	1·0	Absent	15
0·02	0·1	1·0	Present	15
0·02	0·1	2·0	Present	21
0·10	0·1	2·0	Present	6

Table 3. *Extinction changes (ΔE) accompanying the overall reaction of haemoglobin, dithionite and hydrogen peroxide in various concentrations at* pH 8·4

Composition of reaction mixture			Products	CO-haemo-chromogens
Hb (m-equiv./l.)	$Na_2S_2O_4$ (g./100 ml.)	H_2O_2 (m-moles/l.)	($100\Delta E/E$ at 430 mμ)	($100\Delta E$ at 630 mμ)
0·02	0·1	0·5	15	3
0·02	0·1	1·0	20	2
0·02	0·1	2·0	29	3
0·02	0·2	1·0	16	5
0·02	0·2	5·0	37	5
0·02	0·05	1·0	31	2
0·02	0·4	1·0	16	6
0·02	0·8	1·0	16	7
0·02	1·6	1·0	18	8
			($100\Delta E/E$ at 555 mμ)	
0·02	0·1	1·0	18	2
0·05	0·1	1·0	13	9
0·10	0·1	1·0	10	20
0·10	0·1	2·0	17	21

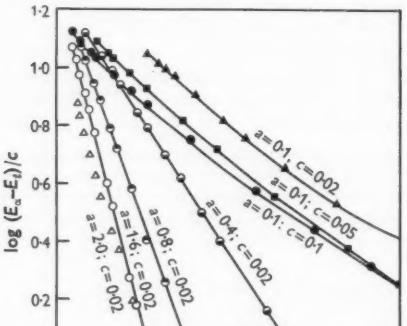


Fig. 5. Effect of $Na_2S_2O_4$ concentration on the rate of reversion of the transient compound to Hb at pH 8·5. $E_\alpha - E_t$ is approximately proportional to the concentration of the transient compound; c = initial Hb concentration (mEq.) and a = $Na_2S_2O_4$ concentration (%); H_2O_2 concentration was mM in all experiments.

In the Soret region, where choleglobin does not absorb appreciably (Lemberg, Holden, Legge & Lockwood, 1942), the final extinction changes are consistent with the reconversion of a single compound, characterized by the 417 mμ maximum, into Hb (Figs. 2*A*, 3). The rates of this reaction with various reactant concentrations at pH 8·4 are shown in Fig. 5. The plots are linear except with the lowest $Na_2S_2O_4$ concentrations. The slope is independent of the pigment concentration and increases with the $Na_2S_2O_4$ concentration up to 1·6 %. The half-reaction time calculated from the slopes decreases from 113 sec. with 0·1 % $Na_2S_2O_4$, to 25 sec. with 1·6 % $Na_2S_2O_4$.

In Fig. 6*A*, *B*, *C*, the extinction changes at the visible maxima of Hb, the intermediate compound and choleglobin during the later stages of the reaction are shown. At different pH values the curves for 545 and 555 mμ accurately reflect the time course of the concomitant extinction changes at 430 mμ (not shown). The end of the life of the intermediate spectrum is indicated by simultaneous minima in the curves for these three wavelengths, i.e. 2·8 min. at pH 8·5, 1·3 min. at pH 7·8 and 0·5 min. at pH 7·0, after which the extinction increases again. These final increases to stable values occur at about the same rate and are complete in the same times at all three wavelengths. These results support the conclusion that the Soret maximum at 417 mμ and the maximum at 545 mμ in the intermediate spectrum are due to a single derivative, and that the final extinction changes in these spectral regions represent the reconversion of this substance into Hb.

At 580 mμ the formation of the intermediate spectrum is associated with a sharp increase of extinction. In the experiment at pH 8·5 (Fig. 6*A*) the extinction at this wavelength subsequently decreases at the same rate as at other wavelengths, whilst the intermediate spectrum persists undistorted but with diminishing intensity. There is an inflexion at 3 min. and the subsequent decrease of extinction occurs at the same rate as the increases at 545, 555 and 430 mμ. At pH 8·5 therefore the intermediate spectrum apparently behaves as a single entity. At pH 7·8 and 7 the extinction change at 580 mμ follow the same general course as at pH 8·5 at an increased rate. At pH 7 (Fig. 6*C*) the initial rise in extinction is less and this may be due to the instability of the intermediate at this pH.

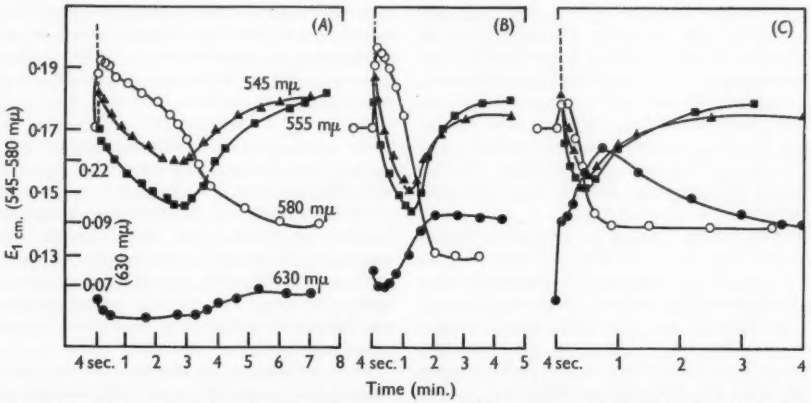


Fig. 6. Spectrokinetic curves for 545, 555, 580 and 630 mμ, for the reaction of 0·02 mEq.-Hb, 0·1% $Na_2S_2O_4$ and 2 mM-H_2O_2. (*A*) pH 8·5; (*B*) pH 7·8; (*C*) pH 7·0.

A discrepancy does arise in that a stable extinction at 580 mμ is reached in 2 min. at pH 7·8 and in 1 min. at pH 7, although at other wavelengths the extinction is still rapidly increasing at these times. A stable Hb spectrum is not obtained until after 5 min. at pH 7·8 and 3 min. at pH 7. Therefore the regeneration of Hb cannot be wholly responsible for the extinction changes at 580 mμ and pH 7–7·8, and the intermediate spectrum cannot be said to behave as an entity throughout the reaction, as it appears to do at pH 8·5.

There is a correlation between extinction changes at 630 and 580 mμ at all three pH values. At 630 mμ the extinction reaches a maximum in 6 min. at pH 8·5, 2 min. at pH 7·8 and 1 min. at pH 7, and the extinction at 580 mμ falls to a constant value in the same times. These results suggest that the maximum at 582 mμ in the intermediate spectrum may be due partly to a choleglobin precursor.

The final decrease of extinction at 630 mμ in the reaction at pH 7 (Fig. 6*C*), which also occurs to a small degree at pH 7·8, may represent the further degradation of choleglobin or the regeneration of Hb.

Effect of carbon monoxide on the reaction. Hydrogen peroxide does not affect the spectrum of carboxyhaemoglobin (HbCO) in $Na_2S_2O_4$ solution at pH 6 and 8·5, and saturation of H_2O_2 solution with CO largely inhibited its reaction with Hb in $Na_2S_2O_4$. The passing of CO into a Hb–$Na_2S_2O_4$ solution immediately after the addition of H_2O_2 during the life of the intermediate spectrum was studied.

Solutions of 0·02 mEq.-Hb in 0·05% $Na_2S_2O_4$ and mM-H_2O_2, both buffered at 8·5, were mixed by passage through the rapid-flow apparatus; under these conditions the lifetime of the intermediate

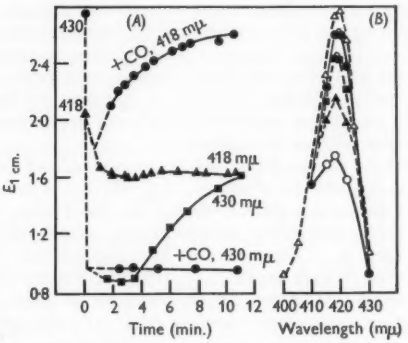


Fig. 7. Effect of carbon monoxide on the reaction of H_2O_2 with Hb in dithionite solution. Hydrogen peroxide was added at time 0 and carbon monoxide passed from 10 to 25 sec. Initial concentrations: 0·02 mEq.-Hb, 0·05% $Na_2S_2O_4$, mM-H_2O_2; pH 8·4. (*A*) Spectrokinetic curves at 418 and 430 mμ with and without CO; (*B*) absorption spectra of reacting mixture in Soret region: ○, 10 sec. (before CO); ▲, 2 min.; ■, 5 min.; ●, 10 min.; △, 1 hr.

compound was 3 min. A portion of the mixture was treated with CO during the period 10–25 sec. after mixing, and transferred to an optical cell which was sealed with liquid paraffin and a coverslip. Change of the extinctions at three wavelengths with time was followed in the sample; similar measurements were made on other samples at other wavelengths. The features of the reaction described below have also been observed in the spectroscope with 0·1 mEq.-Hb, 0·1% $Na_2S_2O_4$ and 2 mM-H_2O_2 at pH 9·5.

The spectrokinetic curves at 418 and 430 mμ, with and without CO treatment (Fig. 7*A*), show

that on the introduction of CO a new reaction accompanied by an increase of extinction at 418 mμ begins at once. During this reaction, the Soret maximum of the intermediate spectrum (Fig. 7*B*) is gradually converted into a much more intense maximum at 419 mμ. These changes can be accounted for by a single reaction, namely the conversion of a derivative characterized by the maximum at 417 mμ into HbCO, $\lambda_{max.}$ 419 mμ, with a half-period of about 2·5 min.

The corresponding changes in the visible region up to 10 min. are shown in Fig. 8*A* and *B*. The initial data in these diagrams correspond to the intermediate spectrum obtained in control experiments. At 535–575 mμ, the extinction changes after the introduction of CO occur at about the same rate as those in the Soret region, and are again consistent with the formation of HbCO ($\lambda_{max.}$ 539, 568 mμ) from the intermediate compound. Another reaction also occurs; a pronounced peak appears at 600 mμ, reaches its greatest intensity in 2 min. and then declines. The subsequent spectral changes at 580–640 mμ, from 10 min. onwards, are shown in Fig. 8*C*. In the course of several hours, the maximum at 600 mμ declines whilst a new peak develops at 628 mμ. Throughout this slow reaction, the absorption spectrum at shorter wavelengths remains almost unchanged.

These results show that in the presence of CO a new unstable intermediate, $\lambda_{max.}$ 600 mμ, is formed in considerable amount during the first 2 min., and is then very slowly converted into carboxycholeglobin, $\lambda_{max.}$ 628 mμ, and the regenerated Hb converted into HbCO, $\lambda_{max.}$ 419, 539, 568 mμ. The spectral absorption of the mixture at intervals during this latter reaction (Fig. 8*C*) shows an isosbestic point at 614 mμ which suggests that the conversion of the new intermediate into carboxycholeglobin is quantitative. At the start of this reaction, that is when the maximum at 600 mμ has reached its greatest intensity, $E_{600\ m\mu}$ is 0·200 and $E_{630\ m\mu}$ is 0·060. The differences between these values and the measured extinctions E_t, at various later times (Table 4), are the same for both wavelengths, and therefore the molar-extinction coefficients of the two substances at their respective maxima are about equal. Since the reaction is not accompanied by significant spectral changes in the regions 536–580 and 400–430 mμ, where typical carboxyhaemoglobin maxima persist unchanged, it also follows that both substances have little specific absorption in these regions.

At intervals during this slow formation of CO-choleglobin from the new intermediate, samples of the reaction mixture were converted into the CO–haemochromogen derivatives. From 16 min. after the introduction of CO the absorption spectra of these derivatives remained constant, and indicated a mixture of proto- and chole-haemochromogen.

Table 4. *Extinction changes at* 600 *and* 630 mμ *during the formation of carboxycholeglobin* (*cf. Fig.* 8*C*)

Time (hr.)	$(0·200 - E_t)$, 600 mμ	$(E_t - 0·06)$, 630 mμ
0·85	0·026	0·025
1·75	0·046	0·044
2·75	0·058	0·056
3·75	0·074	0·072
18·0	0·113	0·111
24·0	0·115	0·114

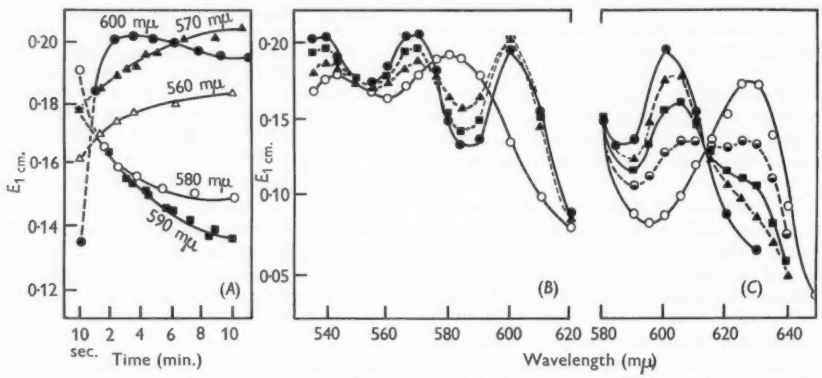


Fig. 8. Effect of carbon monoxide on the reaction of H_2O_2 with Hb in dithionite solution; conditions were as for Fig. 7. (*A*) Spectrokinetic curves from 10 sec. after addition of peroxide; CO was passed during the period 10–25 sec.; (*B*) absorption spectra of reacting mixture: ○, 10 sec. (before CO); ▲, 2 min.; ■, 5 min.; ●, 10 min.; (*C*) absorption spectra of reacting mixture: ●, 10 min.; ▲, 52 min.; ■, 105 min.; ◒, 225 min.; ○, 18 and 24 hr.

Comparison of the absorption spectra of the final products of the reactions with and without CO treatment, and of CO-haemochromogen derivatives of the products, showed that CO greatly increased the choleglobin yield, but did not significantly affect the amount of Hb destroyed.

Effect of hydrosulphide upon the reaction. The reaction of Hb in $Na_2S_2O_4$ solution with H_2O_2 at acid pH produced a pigment with properties akin to those of sulphaemoglobin. It is known that H_2O_2 and sulphides react with Hb in $Na_2S_2O_4$ to form sulphaemoglobin in amounts greater in acid conditions than in alkaline (Michel, 1938). In the reaction under investigation, H_2O_2 is one of the reactants and the other, $Na_2S_2O_4$, is a possible source of H_2S (Bassett & Durrant, 1927). The effect of hydrosulphide on the reaction was therefore studied.

Experiments were made with 0·1 mEq.-Hb solutions in 0·1 % $Na_2S_2O_4$ at pH 6, 8·5 and 9·5, and hydrosulphide (NaSH), which was a 0·5N-NaOH solution saturated with H_2S. The following observations were made with the reversion spectroscope.

(i) At either pH 8·5 or 9·5 (*a*) 0·1 ml. of H_2O_2 was added to 10 ml. of Hb solution. The two bands of the intermediate spectrum, at 588 and 548 mμ, appeared at once, persisted for 1 min. and then gradually fused; the products showed a broad haemoglobin band at 560 mμ and a faint band at 630 mμ. (*b*) When 0·1, 0·3 or 0·5 ml. of NaSH was added to 10 ml. of Hb solution a faint band appeared at once at 620 mμ, but the Hb band remained unaltered. (*c*) When 0·1 ml. of H_2O_2 was added to 10 ml. of Hb solution, followed immediately by 0·1 ml. of NaSH, a strong band appeared at once at 620 mμ and distinct bands at 575 and 541 mμ. There was no change in 10 min. and addition of 30 mg. of $Na_2S_2O_4$ caused no change in 5 min. The solution was red–brown by transmitted light, green–brown by reflected light. (*d*) In a similar experiment, the NaSH was added 90 sec. after the H_2O_2, when the intermediate spectrum was giving way to a Hb spectrum. Immediate observation showed a Hb band and a fairly strong band at 620 mμ, weaker than that obtained in (*c*), much stronger than that obtained in (*b*). (*e*) 0·1 ml. of NaSH was added to 10 ml. of Hb solution, and after rapid observation of the band formed at 620 mμ, cf. (*b*), 0·1 ml. of H_2O_2 was added. Immediate observations showed that the band at 620 mμ had become broader and stronger, and its centre had shifted to 623 mμ; and that the haemoglobin band had been replaced by bands at 575 and

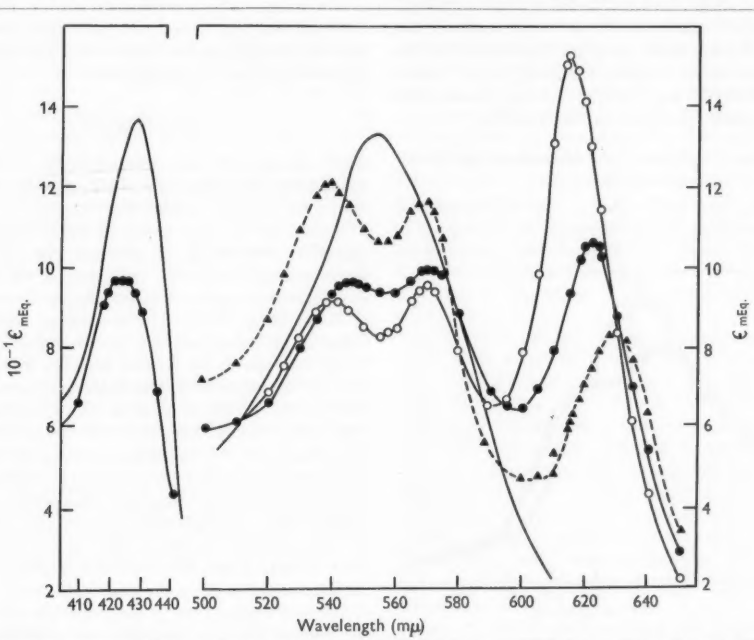


Fig. 9. Absorption spectrum of the products and derivatives of the reaction of 0·1 mEq.-Hb, 0·1 % $Na_2S_2O_4$, mM-H_2O_2 and 5 mM-NaSH at pH 8·5. The ordinates are milliequivalent extinction coefficients referred to the initial Hb concentration. —, Hb; ●, products; ○, products treated with CO; ▲, CO-haemochromogen derivatives of products.

541 mμ. There was no further change after several minutes.

(ii) At pH 6. (*a*) 0·1 ml. of H_2O_2 was added to 10 ml. of Hb solution. A distinct band appeared at once at 626 mμ, but the Hb band remained unchanged: the intermediate spectrum was not observed. (*b*) The addition of 0·1 ml. of NaSH to the products of (*a*) 1 min. after the original addition of H_2O_2 had no effect. (*c*) The addition of 0·1 ml. of NaSH to 10 ml. of Hb solution had no effect.

The absorption spectra of products obtained as in (i) (*c*) above consistently showed a pronounced peak at 622 mμ, shifted to 616 mμ by CO (Fig. 9), but its intensity varied by about 30 % in different preparations under apparently identical conditions. Two maxima at 545 and 570 mμ were usually formed, but were replaced in the course of 30 min. or more by a broad maximum at 555 mμ. In some preparations a single broad maximum at 555 mμ was obtained at once; there was no correlation between the appearance of the two bands in the green and the intensity of the maximum at 622 mμ. There was no sign of an inflexion at 630 mμ in the product spectrum, such as would be expected if appreciable amounts of choleglobin were present, yet in the absorption spectrum of CO-haemochromogen derivatives prepared from the fresh products (Fig. 9) a pronounced maximum at 630 mμ suggests the presence of cholehaem, and the depression of the CO-protohaemochromogen maxima (initial values $\epsilon_{mEq.} = 14{\cdot}4$ at 539 mμ, 14·0 at 569 mμ) shows that some protohaem destruction has occurred.

Reaction of hydrogen peroxide with myoglobin in dithionite solution

The reaction was studied by spectrokinetic measurements at 13 wavelengths in the region of 536–640 mμ from 1 min. after the addition of peroxide until a stable spectrum was obtained. Within 1 min. of the addition of H_2O_2, the myoglobin curve was replaced by a new spectrum with a maximum at 548 mμ, $\epsilon_{mM} = 10{\cdot}5$, and a pronounced shoulder at 585 mμ, $\epsilon_{mM} = 9{\cdot}0$, which persisted without change of shape but with very slowly decreasing intensity for 10 min. and then gradually gave way to a myoglobin curve of slightly reduced intensity (Fig. 10). The intermediate spectrum is indistinguishable from that of metmyoglobin–H_2O_2, and the extinction coefficients indicate that all the myoglobin is converted into this derivative (cf. George & Irvine, 1951). There is no evidence of the presence of cholemyoglobin or any other coloured degradation product. The absorption spectrum of CO-haemochromogen derivatives of the products confirmed that a small proportion of the myoglobin was destroyed in the overall reaction by degradation of the protohaem, but there was no evidence of the presence of any other haem derivatives.

The effect of passing CO into a reacting mixture of myoglobin, dithionite and peroxide, immediately after the addition of peroxide and during the life of the intermediate spectrum, was observed in the spectroscope. The two bands shifted slightly, to 579 and 541 mμ, consistent with the formation of carboxymyoglobin, but there was no evidence of the formation of any other pigment.

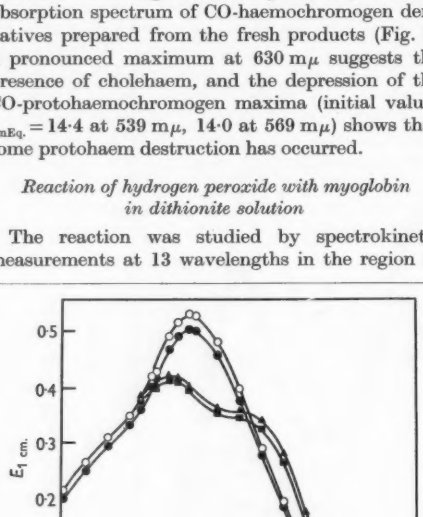


Fig. 10. Spectral absorption of a reacting mixture of 0·04 mEq.-myoglobin, 0·1 % $Na_2S_2O_4$ and 2 mM-H_2O_2, pH 9·5, at intervals after the addition of peroxide: ○, myoglobin, $t = 0$; ▲, 1 min.; ■, 10 min.; ●, products.

DISCUSSION

The results of the experiments confirm the mechanism proposed for the action of oxygen on solutions of Hb containing $Na_2S_2O_4$ (Dalziel & O'Brien, 1957). They point to the formation of an unstable derivative of Hb on the addition of hydrogen peroxide, the existence of which is substantiated by magnetokinetic measurements (Dalziel & Ehrenberg, 1955). The substance with a Soret band at 417 mμ and low paramagnetic susceptibility appears to be formed directly from Hb at a rate which grows with the hydrogen peroxide concentration. It has a lifetime which varies directly with the peroxide concentration and inversely with the dithionite concentration, and is shortened by catalase; it is largely reconverted into Hb at a rate increasing with the $Na_2S_2O_4$ concentration up to a certain limit. It is probably formed by oxidation of, or co-ordination of an oxidizing group with, the iron atoms of Hb and survives during the life of hydrogen peroxide in the reaction mixture.

Known derivatives of haemoglobin which might be formed in the reaction are oxyhaemoglobin (HbO_2), methaemoglobin (Meth), methaemoglobin-hydrogen peroxide (Meth-H_2O_2) and methaemoglobin-hydrosulphide. The last of these may be

dismissed because hydrosulphide did not prolong the life of the intermediate spectrum. For the other three and for the intermediate spectrum, the wavelengths of the absorption maxima and the corresponding $\epsilon_{mEq.}$ are given in Table 5 (Lemberg & Legge, 1949; Dalziel & O'Brien, 1954*a*). Neither HbO_2 nor Meth accounts satisfactorily for the intermediate spectrum. The Soret band of Meth at pH 8·5 is at a distinctly different wavelength, and moves from 405 mμ ($\epsilon_{mEq.} = 165$) at pH 6 to 413 mμ ($\epsilon_{mEq.} = 106$) at pH 10. If the intermediate spectrum is attributed to HbO_2, it must also be assumed, to account for the low extinction coefficients, that 40 % of the Hb is simultaneously converted into products with little specific absorption, and to account for the fact that under some conditions 80 % of the Hb is eventually recovered (Table 3), that one of these products is also reconverted into Hb. Moreover, since the extinction coefficients of the intermediate spectrum are reproducible to within about 5 %, the high proportion of these other products formed must be approximately constant over a wide range of reactant concentrations and pH. These conditions are improbable; and a complicated mechanism would be required to explain the persistence and slow deoxygenation of HbO_2 in the presence of excess of dithionite.

The intermediate spectrum differs significantly from that of Meth-H_2O_2 only in that it shows a maximum at 582 mμ, consistently higher than that at 545 mμ. It could be accounted for by the assumption that in the initial rapid reaction with hydrogen peroxide about 90 % of the Hb is converted into Meth-H_2O_2 and the rest into a derivative with little specific absorption in the Soret region and a maximum at about 580–590 mμ, $\epsilon_{mEq.} \simeq 10$. The degree of reproducibility of the broad, shallow maximum at 582 mμ permits considerable variation of such a small proportion of a secondary product formed under different conditions. A precursor of choleglobin might well exhibit such spectral properties and be formed either simultaneously with Meth-H_2O_2 or very rapidly from it, and persist in a stationary state during the life of peroxide. There was some spectrokinetic evidence that the extinction changes near 580 mμ were associated with choleglobin formation, and in the presence of carbon monoxide a precursor of carboxycholeglobin, $\lambda_{max.}$ 600 mμ, was detected.

Table 5. *Wavelengths of absorption maxima and milliequivalent extinction coefficients (in parentheses) for some haemoglobin derivatives and the intermediate spectrum at* pH 8·5

	Absorption maxima (mμ)		
Oxyhaemoglobin	415 (128)	541 (15)	576 (16)
Methaemoglobin	408 (120)	541 (8·5)	576 (8·0)
Methaemoglobin-hydrogen peroxide	418 (105)	545 (10·5)	582 (8·6)
Intermediate spectrum	417 (90)	545 (9·0)	582 (9·6)

Meth-H_2O_2 has properties consistent with the observed features of the reaction. It has been characterized as an unstable intermediate in the reaction between Meth and H_2O_2 at pH 6–9·5, which results ultimately in the oxidative degradation of part of the protohaem, and the formation at pH 6 of a green pigment distinct from choleglobin (Keilin & Hartree, 1951; Dalziel & O'Brien, 1954*a*, *b*). Until recently, it was assumed to be a ferric peroxide complex (Keilin & Hartree, 1935; Haurowitz, 1935), and if this were so the fact that its formation from methaemoglobin at pH 8·5 is slower than that of the compound from Hb in the presence of dithionite, with the same peroxide concentration, would be difficult to reconcile with the identity of the two compounds. There is reason to believe, however, by analogy with MetMb-H_2O_2 (George & Irvine, 1952, 1954; Theorell & Ehrenberg, 1952) that Meth-H_2O_2 may be a ferryl derivative: its formation from Meth might be represented for example by Fe^{3+} ion $+ H_2O_2 \rightarrow FeO^{2+}$ ion $+ OH + H^+$ ion, and from Hb by Fe^{2+} ion $+ H_2O_2 \rightarrow FeO^{2+}$ ion $+ H_2O$, and there is no reason why the latter reaction should not be faster than the former. Again, the addition of $Na_2S_2O_4$ to a solution of Meth and hydrogen peroxide at pH 8·5 is followed by a sequence of spectral changes similar to those observed in the Hb–$Na_2S_2O_4$–hydrogen peroxide reaction: there is first a slow reaction associated with a general decline of specific absorption, and the Meth-H_2O_2 spectrum is then slowly displaced by a Hb spectrum with a small choleglobin maximum at 630 mμ (Dalziel & O'Brien, 1954*a*). Judged by the rate of increase of extinction at 430 mμ, the rate of Hb regeneration is slower than in the present experiments, but this difference is of uncertain significance in view of the complexity of the reactions.

The life of the intermediate spectrum is associated with a molar paramagnetic susceptibility value of 4500 ± 500 c.g.s.e.m.u. (Dalziel & Ehrenberg, 1955). Values of this order (3000–5000) are common to MetMb-H_2O_2 and -methyl H_2O_2 and the peroxide compounds of types II and III of peroxidase and catalase (Theorell & Ehrenberg, 1952). The susceptibility of Meth-H_2O_2 has not yet been measured, but is likely to be of the same order, since it resembles the myoglobin compound quite closely in spectral and other properties. The reaction between MetMb, $Na_2S_2O_4$ and peroxide at pH 8–9 is accompanied by a sequence of spectral changes similar to those observed with Hb, but differs from the latter in the following respects: (1) the intermediate spectrum in the visible region, $\lambda_{max.}$ 548 mμ, $\epsilon_{mM} = 10·5$, $\lambda_{max.}$ 585 mμ, $\epsilon_{mM} = 9·0$, agreed with

that reported for MetMb-H_2O_2, $\lambda_{max.}$ 549 mμ, $\epsilon_{mM} = 9{\cdot}8$, $\lambda_{max.}$ 585 mμ, $\epsilon_{mM} = 8{\cdot}4$ (George & Irvine, 1951); (2) no detectable amounts of cholemyoglobin were formed in the overall reaction; (3) the proportion of the pigment destroyed by haem degradation was smaller than with Hb under similar conditions; (4) carbon monoxide did not reveal a new intermediate or promote the formation of cholemyoglobin. It is much more likely that myglobin and Hb differ in the rates or mechanism of haem degradation by hydrogen peroxide in the presence of $Na_2S_2O_4$ (cf. Dalziel & O'Brien, 1954*b*) than that they form different derivatives of intact haem under similar conditions.

Although identification of a transient derivative of Hb formed in a complex reaction mixture is not easy, and analogy between Hb and myoglobin must not be over-stressed, the evidence indicates that the intermediate spectrum is due to Meth-H_2O_2 together with a small amount of a substance ultimately converted into choleglobin.

Degradation products

The main bulk of the Hb in the reaction mixture escapes destruction as a consequence of the presence of excess of $Na_2S_2O_4$, and the rest is changed into definable and indefinable products. In alkaline conditions choleglobin is the main recognizable degradation product. But other products with little specific absorption are probably formed, as there is no correlation between the choleglobin formed and the Hb destroyed, a conclusion supported by the fact that passage of carbon monoxide into the reaction mixture increased carboxycholeglobin formation but not haemoglobin destruction. Oxidative break-up of haem is therefore likely and would explain the decline of absorption intensity over the whole spectrum, including 630 mμ, during the life of the intermediate compound.

Formation of choleglobin

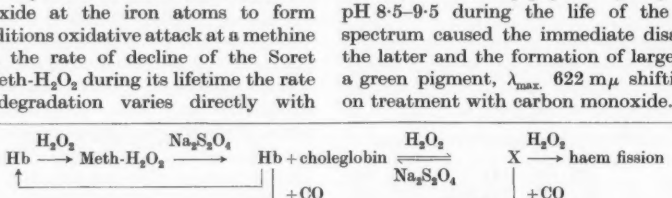
The experimental evidence gives but a sketch of the mode of formation of choleglobin. Since HbCO in $Na_2S_2O_4$ solutions did not yield the intermediate spectrum with hydrogen peroxide nor undergo degradation, it may be concluded that reaction of hydrogen peroxide at the iron atoms to form Meth-H_2O_2 conditions oxidative attack at a methine linkage. From the rate of decline of the Soret maximum of Meth-H_2O_2 during its lifetime the rate of oxidative degradation varies directly with $Na_2S_2O_4$ concentration but is independent of hydrogen peroxide concentration. The yield of choleglobin is similarly affected. These facts may be explained by an oxidation–reduction cycle in which Meth-H_2O_2 is mainly reduced to Hb and partly degraded to choleglobin. The choleglobin undergoes further oxidation by hydrogen peroxide and accumulates only when the latter has been largely destroyed.

After the introduction of carbon monoxide into the reacting mixture, during the life of the intermediate spectrum, the presence of a compound closely related to choleglobin was demonstrated. Spectrokinetic measurements were consistent with the following reactions: (*a*) Meth-$H_2O_2 \rightarrow$ HbCO, half-time approx. 2½ min.; (*b*) X ($\lambda_{max.}$ 580–590 mμ) $\rightarrow$ XCO ($\lambda_{max.}$ 600 mμ), complete in 2 min.; (*c*) XCO $\rightarrow$ CO-choleglobin, lasting several hours. Reaction (*c*) is too slow to involve peroxide.

The tentative mechanism in Scheme I relates these reactions. The greatly increased yield of (carboxy)-choleglobin brought about by carbon monoxide is attributed to the rapid conversion of X into its carbon monoxide derivative, and the resistance of the latter to further oxidative degradation; it may be that X and choleglobin, like Hb, only undergo oxidative degradation in a higher oxidation state or as peroxide derivatives. The conversion of XCO into carboxycholeglobin is quantitative. Foulkes, Lemberg & Purdon (1951) have suggested that cholehaem is derived from pseudohaem, with the grouping $\rangle C{\cdot}CH(OH){\cdot}C(OH)\langle$ which could arise by the addition of hydrogen peroxide at a methine linkage of protohaem, and have shown that it is reduced to protohaem by boiling alkali and dithionite. The intermediate XCO rapidly yields cholehaemochromogen with cold alkali and dithionite, and might be an 'oxidized pseudohaem' derivative, with the grouping $\rangle C{\cdot}CO{\cdot}C(OH)\langle$ which Foulkes *et al.* (1951) believe to be the next stage in the oxidative degradation of Hb.

Sulphaemoglobin

The introduction of hydrosulphide into a reaction mixture of Hb, $Na_2S_2O_4$ and hydrogen peroxide at pH 8·5–9·5 during the life of the intermediate spectrum caused the immediate disappearance of the latter and the formation of large quantities of a green pigment, $\lambda_{max.}$ 622 mμ shifting to 616 mμ on treatment with carbon monoxide. These results



Scheme I

suggest that sulphaemoglobin is formed by the intervention of hydrosulphide at some intermediate stage in the oxidative fission of the methine bridge. Foulkes *et al.* (1951) suggested a structure for the prosthetic group of sulphaemoglobin closely related to that of cholehaem, with the grouping

$$\rangle C\cdot C(SH)\cdot C(OH)\langle$$

in place of a methine bridge. Lemberg & Legge (1949) have postulated the formation of sulphaemoglobin and choleglobin from the same precursor, a peroxide derivative of Hb. The rapidity of the reaction which follows the introduction of hydrosulphide probably accounts for the variations in the sulphaemoglobin yield under apparently identical conditions. Although complete conversion of Hb into sulphaemoglobin has never been achieved, Drabkin & Austin (1935–36) estimated $\epsilon_{mEq.} = 10–11$ at 620 mμ for pure sulphaemoglobin, and Lemberg *et al.* (1942) give $\epsilon_{mEq.} = 16$ at 617 mμ for carbon monoxide-sulphaemoglobin. On the basis of these figures, it would appear that the sulphaemoglobin yield in some of our experiments was close to 100%. This conclusion cannot be accepted, since the spectrum of the product showed a pronounced Soret band, $\lambda_{max.}$ 424, $\epsilon_{mEq.} = 97$, and according to Lemberg *et al.* (1942) carbon monoxide-sulphaemoglobin, like choleglobin, has relatively weak absorption in the Soret region. Moreover, with alkali and dithionite the products apparently yielded a mixture of proto- and chole-haemochromogen, although there was no evidence of the presence of choleglobin in the products themselves. There is evidence that sulphaemoglobin yields protohaemochromogen derivatives (Nijveld, 1943; Lemberg *et al.* 1942).

Choleglobin, sulphaemoglobin and other 'green haemoglobins' such as pseudohaemoglobin (Barkan & Schales, 1937, 1938) and cruoralbin (Holden, 1943, 1945), have generally been obtained by the relatively slow oxidation of Hb or HbO_2 under different conditions. The present work shows that two such derivatives can be obtained in quantity in rapid reactions which follow the successive addition of peroxide and a second reagent to Hb in dithionite solution, provided the second reagent is added during the lifetime of an unstable peroxide derivative of Hb. The results support the general mechanism advanced by Lemberg and his co-workers for these reactions (Lemberg & Legge, 1949; Foulkes *et al.* 1951), although they favour the view that the so-called Meth-H_2O_2, rather than a ferrous peroxide complex (Lemberg, Legge & Lockwood, 1938, 1939, 1941*a*, *b*), is the intermediate involved, and emphasize the importance of the time factor and the use of dilute solutions, in which the life of the peroxide derivative is longest. More detailed studies by means of the compound rapid-flow method of Hartridge & Roughton (1924), whereby a second reagent, e.g. carbon monoxide, hydrosulphide or cyanide, can be introduced at various intervals after the addition of peroxide may clarify the relationship between the various green haemoglobins. The difference between the reactions of Hb and myoglobin with peroxide and dithionite, and the relationship between these reactions and products and those of Meth with peroxide, in which a green pigment distinct from choleglobin is formed (Keilin & Hartree, 1951; Dalziel & O'Brien, 1954*b*), also deserve further study, and may bear upon the mechanism of peroxidase activity.

SUMMARY

1. The reaction between haemoglobin, dithionite and hydrogen peroxide has been investigated by spectrokinetic measurements. The results show that by reaction with the peroxide, haemoglobin is largely converted into an unstable derivative, which persists for a definite period, and is then largely reduced to haemoglobin by the excess of dithionite. In the overall reaction, part of the haemoglobin is destroyed, and small amounts of choleglobin are formed at pH 7–10 and of sulphaemoglobin at pH 6.

2. The intermediate compound is probably methaemoglobin-hydrogen peroxide, which exists in a mixture with a small proportion of a substance subsequently converted into choleglobin.

3. A new unstable intermediate has been detected in the reaction at pH 8·5–9·5 in the presence of carbon monoxide, which is characterized by a maximum at 600 mμ, and is slowly but quantitatively converted into carboxycholeglobin in the presence of excess of dithionite.

4. The addition of hydrosulphide to a reacting mixture of haemoglobin, dithionite and peroxide during the life of the peroxide intermediate at pH 8·5–9·5 at once gives a large yield of a derivative with the spectroscopic properties of sulphaemoglobin.

5. A mechanism is discussed for these reactions.

6. Experiments indicate that myoglobin is temporarily but completely converted into met-myoglobin-hydrogen peroxide on treatment with hydrogen peroxide in the presence of excess of dithionite. Small amounts of pigment were destroyed in the overall reaction, but there was no evidence of the formation of cholemyoglobin or other green pigments.

7. The explanation previously advanced for the action of oxygen on haemoglobin solutions containing dithionite is confirmed.

We wish to thank Mr B. A. Collett for technical assistance in part of this work. We are indebted to Professor Hugo Theorell for a gift of myoglobin. One of us (K. D.) is indebted to the Nuffield Haematology Fund for financial support.

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The Fractionation of Urinary Iodine

1. METHOD OF ANALYSIS*

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The metabolism of radioactive iodine has been the subject of intensive investigation in recent years. This has been concentrated mainly on the production of hormone by the thyroid gland and its subsequent fate in the plasma and tissues, and little has been published about the excretion of the hormone in the urine. It is, however, generally agreed that the greatest proportion of the iodine in urine is present as inorganic iodide, and only a small percentage exists in an organic form. It was to investigate this organic fraction that the present work was undertaken, both to estimate the amount of organically combined iodine and to discover the chemical nature of the products. This present paper describes a method for the investigation of the problem, and subsequent papers will describe the results obtained from the application of this method to the urine of patients receiving large amounts of radioactive iodine for the treatment of disease of the thyroid gland.

Several methods were used in an attempt to separate the organic iodine from the iodide, and most were abandoned. The chief cause of the difficulties encountered was the ease with which iodide is oxidized in an acid solution, leading to the formation of free iodine and the production of artifacts. Acland (1952) has pointed out that biological solutions of iodide can readily produce many artifacts when acidified, and this observation has been amply confirmed. The method finally adopted was the removal of iodide from the urine at an alkaline pH by a column of silver chloride, and the subsequent concentration of the organic iodine by an ion-exchange resin.

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EXPERIMENTAL AND RESULTS

Paper chromatography. Throughout this work chromatography was carried out on Whatman 3 MM paper. This thick paper was chosen since all the material examined contained

large quantities of extraneous non-radioactive matter, which tended to overload thinner paper.

The chromatograms were developed by the descending method, the upper phase of a mixture of *n*-butanol–dioxan–aq. 2N-NH_3 soln. (4:1:5, by vol., Gross, Leblond, Franklin & Quastel, 1950) being used.

Preparation of radioactive compounds. Thyroxine and 3:5-di-iodotyrosine were labelled with radioactive ^{131}I by a modification of the method of Gross & Leblond (1950). The specific activities obtained were initially about 4 $\mu c/\mu g$. Since there is little information about the nature of the iodine-containing compounds of urine, these two compounds were used as models to test the efficacy of the separation procedures.

Location of radioactive compounds and carriers. Positions of radioactivity on paper chromatograms were found either by radioautography on Ilford No-Screen X-Ray film, or by scanning the paper strips in an automatic strip counter. The locations of added carrier compounds were found either by spraying with diazotized sulphanilic acid, or, on chromatograms containing much extraneous material, by the method of Bowden, Maclagan & Wilkinson (1955) as modified by Fletcher & Stanley (1955).

Removal of iodide by silver. Silver forms an extremely insoluble iodide, the solubility product of which is $0{\cdot}32 \times 10^{-16}$, and therefore precipitation with silver should be a highly efficient means for the removal of iodide. The precipitation of iodide by silver has been used previously by several authors, but some doubt seems to exist about the solubility of the silver salts or complexes of thyroxine and di-iodotyrosine. Thus Albert, Rall, Keating, Power & Williams (1949), following the procedures of Foster & Gutman (1930) and Gross & Leblond (1947), precipitated the iodide from urine with $AgNO_3$ or Ag_2SO_4 in an acid solution and found little loss of di-iodotyrosine. Brown & Jackson (1954), on the other hand, fractionated plasma with silver phosphate, and found that thyroxine was unprecipitated whilst still attached to native globulin, but on precipitation and removal of the plasma proteins with methanol the free thyroxine was quantitatively removed with the silver phosphate. Thyroxine is known to form chelated derivatives with many metals (Gemmill & Plunkett, 1952; Lardy, 1955) and it seems possible that silver, which readily forms complexes, might chelate with thyroxine to form an insoluble product.

Preliminary experiments had shown that the direct precipitation of iodide from urine, at an alkaline pH, was impracticable since the silver iodide formed a suspension almost colloidal in nature and impossible to filter clear. It was, however, found that percolation of a solution of iodide through a column of AgCl removed the iodide, and therefore the influence of AgCl upon thyroxine was investigated.

Table 1. *Solubility of silver thyroxine in various media*

For experimental procedure see text.

Composition of soln.	pH	Solubility of thyroxine ($\mu g./ml.$) Expt. 1	Expt. 2
Water	7	5·1	7·1
50% ethanol	7	29·0	25·6
50% ethanol	9	—	81·5
50% ethanol; NaCl (5 mg./ml.)	9	180·0	174·0
50% ethanol; NaCl (10 mg./ml.)	7	191·0	—
50% ethanol; NaCl (10 mg./ml.)	8	178·0	—
50% ethanol; NaCl (10 mg./ml.)	8·5	161·0	219·0

Labelled thyroxine (225 μc) was mixed with a solution of 10·8 mg. of unlabelled thyroxine in 0·001N-NaOH. A volume (2 ml.) of 0·1N-$AgNO_3$ was added and the solution kept for 2 hr. The precipitate of silver thyroxine was centrifuged and the supernatant discarded. The precipitate was washed twice with 0·001N-NaOH and four times with water. A small quantity of the precipitate was then suspended in 10 ml. of various media and left overnight with constant shaking. The solutions were centrifuged and portions of the clear supernatants counted. The results of two such experiments are given in Table 1. From these results it would appear that there would be no loss, by the formation of an insoluble silver salt, of thyroxine in the concentrations usually encountered in biological fluids, provided the optimum conditions of Table 1 were used. A similar series of investigations on the solubility of di-iodotyrosine was not performed since experiment showed that 100 ml. of urine would dissolve at least 1 mg. of di-iodotyrosine in the presence of AgCl.

Preparation of silver chloride column. Silver chloride was precipitated from $AgNO_3$ by the addition of HNO_3 and NaCl. The resulting solid was washed completely free from acid by decantation and poured in a slurry, in a solution of 50% ethanol containing 1% NaCl at pH 8·5, into a glass tube closed by a plug of glass wool and a tap. This was packed to give a flow rate of 0·2–1 ml./min., the slower rate being for a column 1·5 cm. × 15 cm., the faster for a column 2·8 cm. × 22 cm. The column was jacketed with dark translucent paper to reduce photodecomposition of the AgCl.

Removal of iodide from urine

To test the efficacy of the column for the removal of iodide the following solution was made up. Radioactive ^{131}I and carrier ^{127}I were added to normal urine and the pH was adjusted to 8·5. An equal volume of ethanol was added and the precipitated phosphates were filtered off and washed with 50% ethanol, the washings being added to the filtrate. The total activity of the solution was determined, and the solution was allowed to flow slowly through a column of AgCl prepared as above. When nearly all the solution had run through, a further 50 ml. of 50% ethanol was added to the column to wash the remaining urine down the tube. The activity of the percolate was found. The results of experiments on the recovery of iodide from urine are given in Table 2 (*a*). It can be seen that the removal is almost complete, and examination of the column showed that the iodide was removed in the first few centimetres of the column, as judged by the yellow band of the AgI.

Recovery of [^{131}I]*thyroxine and* [^{131}I]*di-iodotyrosine*

The recovery of labelled thyroxine and di-iodotyrosine was found in a manner similar to that used for iodide except that no stable iodide was

added and no additional thyroxine or di-iodotyrosine was present, apart from that in the radioactive preparations. The results obtained are given in Table 2 (*b*, *c*). Chromatography of the solutions after passage through the column showed only the added radioactive compound to be present, plus small traces of iodide.

Separation of [^{131}I]iodide, [^{131}I]thyroxine and [^{131}I]di-iodotyrosine

Table 3 shows the results obtained when mixtures of radioactive iodide, thyroxine and di-iodotyrosine were passed through the column. The percentages of organic activity in the urine before and after the column treatment are given.

Concentration of organic iodine from urine

The urine, after passage through the AgCl column, still contained nearly all the salts and extraneous matter originally present. It was necessary therefore that the organic iodine be concentrated before it was suitable for study. It seemed probable that any metabolites of the thyroid hormone excreted in urine would still retain one or two benzene rings and that they should therefore be capable of separation by adsorption. Experiments with various activated charcoals showed that these materials led to both extensive decomposition and irreversible adsorption of thyroxine and di-iodotyrosine. Use was therefore made of an ion-exchange resin as an adsorbing material.

Table 2. *Recoveries of [^{131}I]iodide, [^{131}I]thyroxine and [^{131}I]di-iodotyrosine after passage through a column of silver chloride*

Size of columns (cm. × cm.)	Compounds added	Amount added (μc)	Urine volume (ml.)	Recovery (%)
1·5 × 15	(*a*) Iodide [Each urine contained 1% (v/v) of N-NaI]	123	160	0·035
1·5 × 15		43	25	0·71
1·5 × 15		200	50	0·17
2·8 × 22		635	500	0·038
1·5 × 15	(*b*) Thyroxine	3·9	40	97
1·5 × 15		2·1	48	97
2·5 × 22		71·0	450	84
1·5 × 15	(*c*) Di-iodotyrosine	2·7	45	92
1·5 × 15		12·6	100	94
2·5 × 22		16·0	350	82

Table 3. *Separation of [^{131}I]thyroxine and [^{131}I]di-iodotyrosine from [^{131}I]iodide by a column of silver chloride*

Each urine contained 1% (v/v) of N-NaI.

Size of column (cm. × cm.)	Compounds added	Amount (μc)	Radioactivity of organic iodine (%) Added	Recovered	Urine vol. (ml.)
1·5 × 15	Iodide	40	3·2	3·3	40
	Thyroxine	1·3			
1·5 × 15	Iodide	8·9	8·3	7·9	120
	Thyroxine	0·8			
2·8 × 22	Iodide	289	11·6	11·9	500
	Di-iodotyrosine	37·3			
	Thyroxine	0·5			

Table 4. *Recovery of thyroxine, di-iodotyrosine and iodide in the various fractions obtained by treatment of urine with an ion-exchange resin*

Each column represents a separate experiment. The recovery is given as percentage of the original radioactivity in the urine. For experimental details see text.

Radioactive compound added to urine	Thyroxine	Di-iodotyrosine	Iodide
Filtrate after adsorption on resin	2·4	22	82
Filtrate after acid wash	0·1	5	11
Filtrate after alkaline elution	92·0	66	4
Filtrate after alkaline wash	2·3	4	0·5
Residue left on resin	3	3	2

Urine (500 ml.) containing either labelled thyroxine or di-iodotyrosine was adjusted to pH 4 with 10N-HCl, and cooled in an ice bath to 0–4°. A settled suspension (100 ml.) of Zeo-Karb 215 (200–400 mesh, Na form) was added and the mixture stirred continuously in the ice bath for 3 hr. The resin was filtered, and again stirred with 250 ml. of water at pH 4 for a further hour, and the washing fluid was filtered off. The resin was then stirred at room temperature for 2 hr. in 500 ml. of a mixture ethanol–aq. 2N-NH_3 soln. (1:1, v/v). After filtration the resin was finally washed with a further 250 ml. of ethanol–aq. 2N-NH_3 soln. The recovery of thyroxine and di-iodotyrosine in the various fractions is given in Table 4. Chromatography of these fractions showed only the added compound to be present. Table 4 also shows the results obtained when a solution of [^{131}I]iodide in urine was subjected to the same procedure.

Extraction with butanol

By adsorbing the organic iodine on to the resin, the greater part of the interfering substances in the urine was removed, but the alkaline eluate still contained too much material for paper chromatography of the whole solution. The combined alkaline elution fractions were therefore evaporated *in vacuo* to approx. 15 ml. and extracted four times at pH 2 with equal volumes of *n*-butanol. The pooled butanol extracts were then neutralized with conc. NH_3 soln. and evaporated to dryness. The residue proved suitable for the isolation of the organic iodine by paper chromatography.

Chromatography of the concentrated organic iodine

The residue after the evaporation of the butanol was dissolved in a minimum quantity of ethanol–aq. 2N-NH_3 soln. (1:1, v/v). This solution was then applied in a band along the top of two sheets of chromatography paper 15 cm. × 45 cm. The chromatogram was developed overnight, and, after drying, areas of radioactivity were located by radioautography. The radioactive bands were cut out of the paper and eluted overnight. Portions of these eluates were rechromatographed with added carrier compounds and, by the correspondence of the radioautographic spot and the stained area, were shown to contain only the compounds originally added to the urine.

DISCUSSION

By using a column of silver chloride, the iodide in urine may be removed whilst still retaining good recoveries of added labelled compounds. Table 2 (*a*) shows that iodide is not completely removed from the urine, and the amount flowing through the column is variable; in no case, however, did the amount of unextracted iodide exceed 0·8 %, and it is felt that this amount is insignificant. Table 2 (*b*, *c*) shows that the recoveries of added labelled compounds are good, although it seems that the recovery diminishes with increased time of passage through the column. No attempt has been made to increase this recovery since it is probably due to decomposition of the compounds, and further washing of the column elutes only insignificant amounts of radioactivity. When the recovery of organic iodine, in the presence of iodide, is considered, it is found that there is agreement between that recovered and the amount originally added, although this is to some extent fortuitous since the small leak-through of iodide will counterbalance the loss of thyroxine or di-iodotyrosine.

Although it has been shown that the method of analysis is valid when applied to thyroxine and di-iodotyrosine dissolved in urine, it is not necessarily so for urine containing various other metabolites. The form in which iodine is excreted in the urine is almost unknown, and it is possible that compounds are excreted whose behaviour with silver is different from thyroxine. The daily excretion of iodine by persons on a normal diet is approximately 100 μg., and of this it seems that less than 20 % would be in an organic form. The method here described is applicable to a maximum concentration of thyroxine of about 150 μg./ml. and would only fail if the concentration in the urine were to exceed this, or a compound were excreted whose silver salt was some thousand times less soluble than that of thyroxine. Both of these hypotheses are unlikely. It is possible, however, that a compound might be chemically altered during its passage through the column. Silver chloride in an alkaline solution is an oxidizing agent, and examination of the column after the treatment of urine shows that some of the silver chloride has been reduced to metallic silver, an effect that increases sharply with an increase in the pH of the percolating fluid. Therefore a compound with an easily oxidizable group might emerge from the column in an oxidized form, and comparison with the untreated urine would be necessary to determine the original structure of the compound.

The concentration of the activity after the removal of iodide is in several ways unsatisfactory. While thyroxine is recovered almost completely in the alkaline eluate from the resin, much di-iodotyrosine is lost during the adsorption stage. Chromatographic separation, on ion-exchange resins, of these two substances in urine has been attempted, but with less success than with the method discussed here. The pH of 4 used during the adsorption appears to be optimum, and lengthening the time of adsorption does little to improve the recovery of di-iodotyrosine. Since this part of the separation procedure is intended only to

concentrate the urinary iodine for chemical study, it is not necessary that it should be quantitative, but an increase in the recovery of di-iodotyrosine and a better separation of the activity from non-radioactive matter are desirable. In its present form, however, the method has been used in a study of the organic iodine compounds of urine, the results of which are to be published later.

SUMMARY

1. A method for the quantitative estimation of small amounts of ^{131}I-labelled thyroxine and di-iodotyrosine in the presence of large amounts of [^{131}I]iodide in urine is presented.

2. Iodide is removed as insoluble silver iodide by the use of a column of silver chloride.

3. Under certain conditions thyroxine and di-iodotyrosine are not removed from the urine by the column.

4. Thyroxine and di-iodotyrosine are concentrated from the urine by adsorption on an ion-exchange resin.

5. The eluate from the resin, after extraction into butanol, is suitable for paper chromatography.

I should like to acknowledge the constant co-operation and helpful criticism of E. E. Pochin, M.D., F.R.C.P., during the course of this work.

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The Fractionation of Urinary Iodine

2. METABOLITES EXCRETED DURING TREATMENT OF CARCINOMA OF THE THYROID*

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In the literature it is generally agreed that the major proportion of the iodine excreted in the urine is in the form of inorganic iodide. Albert & Keating (1949) fractionated the urine of patients receiving labelled thyroxine orally. They found that 85% of the urinary activity behaved as iodide, the remainder as 3:5-di-iodotyrosine. No chemical identification was attempted apart from the separation procedure. Later Benua, Albert & Keating (1952) repeated this work after giving [^{131}I]thyroxine intravenously. Their findings were similar, save that shortly after the injection they found a definite proportion of the activity, from 0 to 20%, in the thyroxine fraction. This proportion diminished with time, and they concluded that 24 hr. after the dose about 90% of the activity of the urine was present as iodide.

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Taurog (1955), in a review of the excretion of the thyroid hormone, attaches little importance to urinary iodine, but in a paper (Taurog, Briggs & Chaikoff, 1952) on the hepatic formation of the glucuronide of thyroxine by thyroidectomized rats, he regards urinary excretion as 95% iodide and possibly 5% glucuronide. On the other hand Roche, Michel & Tata (1954*a*), giving very large amounts of radioactive thyroxine (approx. 3 mg.) to rats, claim that 40% of the radioactivity present in the urine is in the form of tetraiodothyropyruvic acid, i.e. thyroxine which has been oxidatively deaminated. In contrast with this the same workers (Roche, Michel & Tata, 1954*b*) state that the corresponding keto acid derived from tri-iodothyronine appeared in only minute quantities in the urine, the faeces being the main excretory path for this compound. Tong, Taurog & Chaikoff (1954) found that liver and kidney slices could produce 3:5-di-iodo-4-hydroxyphenylpyruvic acid and 3:5-di-iodo-4-hydroxyphenyl-lactic acid from di-iodotyrosine,

although in urine they found that the iodine from di-iodotyrosine was 90% present as iodide, the remaining 10% not being investigated. Foster & Gutman (1930) fed large amounts of di-iodotyrosine to rabbits and isolated from their urine a compound identified as 3:5-di-iodo-4-hydroxyphenyl-lactic acid. Stanbury and his collaborators (Stanbury, Kassenaar, Meijer & Terpstra, 1956*a*; Stanbury, Meijer & Kassenaar, 1956*b*; Querido, Stanbury, Kassenaar & Meijer, 1956) have investigated the metabolism of di-iodotyrosine and 3-monoiodotyrosine in a series of patients. In most of these they found that di-iodotyrosine was rapidly deiodinated, the iodine appearing in the urine as iodide with only a small fraction, about 10% of the urinary iodine, in the form of di-iodotyrosine; these results were similar to those obtained by Johnson & Albert (1951) in the rat. Monoiodotyrosine was even more rapidly deiodinated, with no monoiodotyrosine appearing in the urine. In a group of people with goitre, however, they found that both monoiodotyrosine and di-iodotyrosine appeared in the urine after the injection of these substances, accompanied by a substance tentatively identified as 3:5-di-iodo-4-hydroxyphenyl-lactic acid and traces of other unknown compounds. They suggested as an explanation of their results that in this second group a deiodinase for di-iodotyrosine and monoiodotyrosine was lacking, and that this led to the appearance of these compounds and their metabolites in the urine.

There is thus little agreement about the form of iodine metabolites in the urine, except that a large proportion is iodide. Quantitatively, however, more evidence is available, and several studies have been made on the rate of excretion of tracer doses of various iodinated compounds. Albert, Rall, Keating, Power & Williams (1949) gave labelled rat thyroglobulin and thyroxine orally to two myxoedematous patients, both before and after therapy with thyroxine. When thyroglobulin was given, 20% of the radioactive iodine was excreted in 12 hr. and 50% in 60 hr. When thyroxine was given only 25% was excreted in 72 hr. by the untreated patient and 45% by the treated patient. The initial phase of the excretion in the latter was also slower than in the patient receiving thyroglobulin. It is probable that, since the thyroglobulin was given by mouth, it was hydrolysed in the gut to the constituent amino acids, and it was these which were being absorbed. If so, this treatment was equivalent to giving labelled thyroxine plus mono- and di-iodotyrosine, and, in view of the rapid breakdown and excretion of the two latter compounds, the differences between the initial excretion rates of thyroglobulin and thyroxine can be explained. Another point is, however, pertinent here. The thyroglobulin used was rat thyroglobulin, obtained from a rat given a dose of radioactive iodide, and the thyroxine contained in it would presumably be labelled equally on each of the benzene rings. The radioactive thyroxine, on the other hand, was prepared by iodination of 3:5-di-iodothyronine and would therefore be labelled only on the ring distal to the side chain. This difference is important when the excretion rates of labelled compounds are considered. Roche, Michel & Tata (1952) injected radioactive thyroxine labelled (*a*) in the 3:5 positions and (*b*) in the 3′:5′ positions, into thyroidectomized rats, and measured the rate of excretion of radioactivity. They found that excretion was faster from the 3′:5′-labelled thyroxine than from the 3:5-labelled. In view of this work, it must be expected that endogenously labelled hormone, labelled equally in both rings, will appear to be metabolized differently from hormone labelled either by synthesis from 3:5-di-iodothyronine or by exchange. [From Harington's (1927) work on the extreme difficulty or impossibility of iodinating the thyronine molecule in the 3:5 positions, and from the failure of experiments designed to label 3:5-di-iodothyronine by exchange, it is probable that the iodine atoms in the 3:5 positions are not exchangeable with free iodine.] Furthermore, it is possible that a search for products of thyroxine degradation would be rendered useless if the only label on the molecule were in the 3′:5′ position and was lost during metabolism. Many workers (Gross & Leblond, 1951; Flock & Bollman, 1954; Pitt-Rivers, Stanbury & Rapp, 1955) have shown that thyroxine can be deiodinated to tri-iodothyronine by various tissues, and it has been postulated (Gross & Pitt-Rivers, 1953; Kalant, Lee & Sellers, 1955; Thibault & Pitt-Rivers, 1955) that some deiodinated form of thyroxine is in fact the active constituent of the thyroid hormone.

From the foregoing it seems probable that, during the metabolism of thyroxine, the iodine atoms are lost, those in the distal ring disappearing first. There is, however, practically no further information on the fate of the thyroid hormone after it has exerted its metabolic effect. The present study was undertaken to discover if any fragments of the various iodinated molecules present in the plasma were excreted in the urine, and also to estimate the quantities of such fragments.

EXPERIMENTAL

The method of analysis used has been described in the previous paper (Fletcher, 1957). The column for this work measured 2·8 cm. × 42 cm., and was packed with AgCl to give a column approximately 22 cm. high, with a flow rate of about 1 ml./min.

Collection and preservation of urine. Material used for the investigation was the urine of patients receiving radioactive-iodine treatment for carcinoma of the thyroid gland. The

course of such treatment may be divided into two stages. In the first the normal thyroid tissue is destroyed by an 'ablation' dose of radioactive iodine, usually of about 80 mc. After destruction of the normal tissue there is often enhanced uptake of radioactive iodine by the abnormal carcinomatous cells, and treatment is continued by succeeding 'therapy' doses of radioactive iodine of 150 mc. The daily excretion of radioactivity after each treatment is shown in Fig. 1. Fig. 1 (*a*) shows the excretion after an ablation dose of radioactive iodine. There are two phases: (1) an initial high output of activity decreasing daily, due to the excretion of unchanged iodide; and (2) a second rise and fall. This latter peak of activity is consequent upon a release of large quantities of organically bound iodine, from the thyroid cells breaking down under irradiation, and may be observed at various time intervals after the dose, usually 5–7 days. Fig. 1 (*b*) illustrates the excretion of activity from a patient with little functioning tissue. The initial fall is more sustained and finally only a small quantity of activity is excreted, this small quantity presumably being derived from a correspondingly low amount of organically bound iodine in the plasma. The two graphs cited are extreme examples of the two modes of excretion. The secondary peak in Fig. 1 (*a*) is not necessarily so marked, and, on the other hand, the urine from a patient with large amounts of functioning neoplastic tissue may contain far less of the dose in the early phase than is shown in Fig. 1 (*b*), and may even show the later peak typical of patients undergoing thyroid ablation. Urine therefore has usually been collected on the sixth day after a dose of radioactive iodine, since it is on about this day that the highest quantity of iodine, from organic iodine in the plasma, will be present. Urine collected on other days has, however, also been investigated. The plasmas of many of the patients have been examined chromatographically and electrophoretically on the sixth day after a dose.

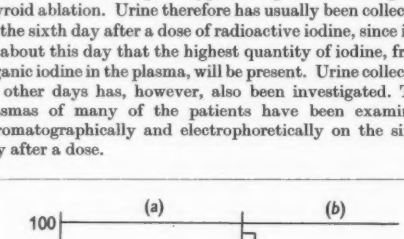

Fig. 1. Daily excretion of radioactive iodine, expressed as a percentage of the dose in patients receiving radioactive iodine for (*a*) ablation of the thyroid gland, (*b*) destruction of residual neoplastic tissue.

A 24 hr. specimen of urine was collected. To avoid possible decomposition of any iodine metabolites or the production of artifacts by the oxidation of iodide, the urine immediately after being passed was poured into a 2·5 l. bottle containing 10 ml. of 40% NaOH, 10 ml. of N-NaI and 1 g. of Na_2SO_3, which was kept in a refrigerator. Initially the pH of the solution in the bottle was probably high (pH 12), but after the 24 hr. specimen had been collected the pH was usually about 10.

Estimation of organic iodine. The 24 hr. specimen was shaken to suspend the precipitated phosphates and 500 ml. was taken for analysis. A small portion was put aside for counting, to determine the initial activity of the urine. To the 500 ml. were added 500 ml. of ethanol and sufficient HCl or NaOH to bring the pH to 8·5. The solution was warmed to about 40° to coagulate the precipitate, and filtered. The residue was washed with 50% ethanol, the washings being added to the filtrate. The precipitate contained a negligible quantity of radioactivity. The clear solution was then put into a large separating funnel attached to the AgCl column and allowed to percolate through the column overnight. When all the solution had drained through, the funnel was washed with 100 ml. of 50% ethanol at pH 8·5, the washings being passed through the column to elute the remaining urine held by the column. The total volume of the percolate was measured, and its activity determined by counting a portion of it. This activity has been taken to be the organic activity of the urine, and, after correction for radioactive decay, has been expressed as a percentage of the original activity of the urine.

Recovery of silver chloride. After the column had been used it was extruded from the tube and, after removal of the top few centimetres containing the bulk of the radioactive AgI (about 50 g.), dissolved in sodium thiosulphate and filtered. To the filtrate was added 100 g. of glucose and 100 ml. of 40% NaOH. On gentle warming for 24 hr. silver was precipitated, and collected by filtration and washed. The silver was dissolved in 33% HNO_3 and filtered, and the resulting solution used to prepare pure AgCl for another column by the addition of excess of a solution of NaCl.

Isolation of organic iodine. The effluent from the column was evaporated, *in vacuo*, at less than 40°, to about 100 ml. to remove the ethanol, and then diluted to approx. 400 ml. It was then subjected to the resin treatment (Fletcher, 1957). The final concentrate was extracted with *n*-butanol, and the butanol extract chromatographed on two sheets of Whatman 3MM paper 15 cm. × 45 cm.

Chromatography. Portions of all the fractions obtained during the separation procedure were chromatographed with *n*-butanol–dioxan–aq. 2N-NH_3 soln. (4:1:5, by vol.) and radioautographed to check that no component was being lost completely. The chromatograms of the final concentrated extract were radioautographed to determine the positions of the various radioactive compounds. The required compound was eluted from the chromatogram and rechromatographed in *n*-butanol–ethanol–water (5:1:4, by vol.). Elution of the bands produced in this second chromatogram gave material which was sufficiently pure for chromatographic comparison with known compounds.

Identification of compounds. A portion of the isolated radioactive compounds was mixed with approx. 100 μg. of a known compound and chromatographed in *n*-butanol–dioxan–aq. NH_3 soln. The pure compound was also run on the same sheet. The chromatogram was radioautographed,

and the position of the reference compound found by spraying with diazotized sulphanilic acid. Coincidence of the radioautographic image and the stained spot was taken to indicate tentative identity of the compounds. To confirm this, the unknown and the suspected known compounds were chromatographed in four different solvents. Coincidence in all four solvents was taken as evidence for the identity of the unknown with the known compound.

Reference compounds. Thyroxine, 3:5:3′-tri-iodothyronine and 3:5-di-iodothyronine were obtained from Glaxo Laboratories Ltd., and were recrystallized before use. 3:5-Di-iodotyrosine was obtained from B.D.H. Laboratories Ltd.

3:5-Di-iodo-4-(3-iodo-4-hydroxyphenoxy)phenylacetic acid, 3:5-di-iodo-4-(3:5-di-iodo-4-hydroxyphenoxy)phenylacetic acid, 3:5-di-iodo-4-(3-iodo-4-hydroxyphenoxy)-phenylpyruvic acid and 3:5-di-iodo-4-(3:5-di-iodo-4-hydroxyphenoxy)phenylpyruvic acid were the gift of Dr R. Pitt-Rivers.

3:5-Di-iodo-4-hydroxyphenyl-lactic acid and 3:5-di-iodo-4-(3:5-di-iodo-4-hydroxyphenoxy)phenyl-lactic acid were synthesized by the method of Saul & Trikojus (1948).

3:5-Di-iodo-4-hydroxyphenylpyruvic acid was synthesized by the method of Tong *et al.* (1954). (This compound was not obtained pure despite repeated recrystallization. However, only one of the spots on a chromatogram of it gave the reactions of an α-keto acid and this was taken as the reference spot.)

3-Monoiodotyrosine was synthesized by the iodination of tyrosine.

RESULTS

Percentage of organic iodine of urine. Table 1 shows the percentage of organic activity found in the urine of patients undergoing ablation of the thyroid gland. Table 2 shows the percentage of organic activity found in the urine of patients with no thyroid, but with uptake in carcinomata.

Isolation of urinary iodine compounds. Fig. 2 shows three chromatograms of the final concentrate of 500 ml. of urine from three different patients. It can be seen that there is a broad similarity between these and they are in fact typical of the chromatograms obtained. The greatest part of the organic activity is found in two compounds, A and B, running very close together near the origin. By counting a chromatogram of a small portion of the concentrate, it has been found that 80–90 % of the

Table 1. *Percentage of the total iodine as organic iodine in the urine of patients undergoing ablation of the thyroid gland*

Numbers in parentheses refer to the day after the ablation dose on which the urine was collected.

Patient	Total activity of 500 ml. of urine (μc)	Organic activity (μc)	Organic percentage
Cr. (2)	3290	81·7	2·5
Cr. (5)	964	84·6	8·8
Cr. (6)	865	62·7	7·3
Ta. (4)	919	29·4	3·2
Ta. (5)	531	34·6	6·5
Ta. (6)	169	12·9	7·6
Ev. (4)	485	13·9	2·8
Mi. (6)	185	15·0	8·1
Be. (6)	255	23·1	9·1

Table 2. *Percentage of the total iodine as organic iodine in the urine of patients with no thyroid but with uptake in carcinomata*

Numbers in parentheses refer to the day after the dose on which the urine was collected.

Patient	Total activity of 500 ml. of urine (μc)	Organic activity (μc)	Organic percentage
Ma. (2)	5090	50·6	1·0
Ma. (3)	2320	19·2	0·83
Ma. (4)	432	4·8	1·4
Ke. (2)	4720	2·0	0·04
Ke. (6)	52	1·9	3·5
Ju. (3)	1426	3·4	0·23
To. (5)	3190	484	15·1
Ev. (7)	1081	73·8	6·8

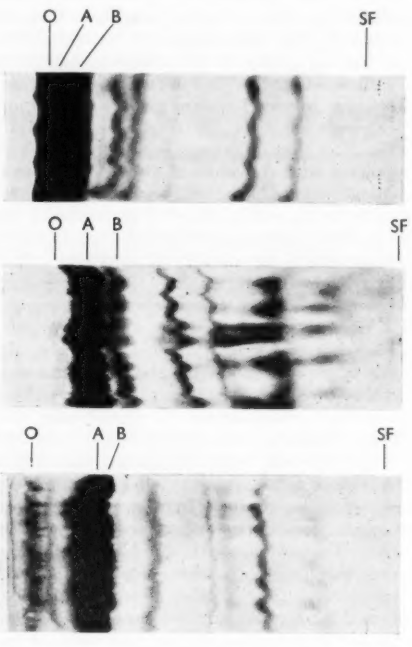

Fig. 2. Final chromatograms of the organic fraction obtained from the urine of three different patients. O, Origin; SF, solvent front. Bands A and B, containing the bulk of the radioactivity, were identified as 3:5-di-iodotyrosine and 3:5-di-iodo-4-hydroxyphenyl-lactic acid.

Table 3. *R_F values of reference compounds*

The descending method with Whatman no. 3MM paper was used. Solvents: I, *n*-butanol–dioxan–aq. 2N-NH_3 soln. (4:1:5, by vol.); II, *n*-butanol–ethanol–water (4:1:5, by vol.); III, *n*-butanol–2N-acetic acid (1:1, v/v); IV, collidine–water (1:1, v/v). Time of run, 16 hr.

Compound	R_F value in solvent			
	I	II	III	IV
3-Monoiodotyrosine	0·20	0·42	0·50	0·63
3:5-Di-iodotyrosine	0·18	0·26	0·60	0·49
3:5-Di-iodo-4-hydroxyphenylpyruvic acid	0·21	0·29	0·86	0·78
3:5-Di-iodo-4-hydroxyphenyl-lactic acid	0·21	0·43	0·91	0·76
Iodide	0·30	0·29	0·21	0·71
3:5:3′-Tri-iodothyronine	0·70	0·95	0·89	0·93
Thyroxine	0·60	0·93	0·92	0·84
3:5-Di-iodo-4-(3-iodo-4-hydroxyphenoxy)phenylacetic acid	0·74	0·84	0·96	0·91
3:5-Di-iodo-4-(3:5-di-iodo-4-hydroxyphenoxy)phenylacetic acid	0·65	0·84	0·97	0·92
3:5-Di-iodo-4-(3:5-di-iodo-4-hydroxyphenoxy)phenyl-lactic acid	0·61	0·80	0·96	0·93
3:5-Di-iodo-4-(3-iodo-4-hydroxyphenoxy)phenylpyruvic acid	0·76	0·85	0·95	0·96
3:5-Di-iodo-4-(3:5-di-iodo-4-hydroxyphenoxy)phenylpyruvic acid	0·66	0·87	0·96	0·98

total activity resides in these two compounds, the remaining activity being distributed between the various compounds separating along the length of the chromatogram. Initially the investigation of the nature of the urinary iodine was devoted to the two compounds constituting the bulk of the organic radioactivity. After purification, these two compounds were run with known compounds. Table 3 gives the R_F values of the reference compounds after chromatography in four solvents. It can be seen that in solvent I the compounds separate into two distinct groups: the single-ring compounds having R_F values of about 0·2 and the two-ring compounds with R_F values of about 0·6. Since the two main compounds from the urinary chromatograms gave R_F values of 0–0·2 in solvent I, they were purified and re-run with carrier amounts of all the single-ring compounds in solvents I–IV. It was found that compound A, the slower of the two unknowns in solvent I, was chromatographically identical with di-iodotyrosine, and compound B with 3:5-di-iodo-4-hydroxyphenyl-lactic acid.

Similar experiments with the other unknowns from the urine chromatograms did not yield such unequivocal information. In these only small amounts of activity were available, and comparison between different chromatograms was difficult since there was no certainty that the same compound was being studied. In no case, however, was thyroxine, tri-iodothyronine, tri-iodothyroacetic acid or tetraiodothyroacetic acid found. Identity of some of the spots with other reference compounds was equivocal, and further work is needed to confirm this. Experiments were also carried out to hydrolyse the various components, and to treat them chemically in other ways, but again no useful information was obtained. From this work it appears that most of the minor iodine-containing compounds of urine are not recognized thyroid metabolites.

DISCUSSION

The results in Tables 1 and 2 show that the amount of organically combined iodine in urine reflects the uptake by functioning tissue of the patient. Thus in the group with no normal thyroid tissue (Table 2), patients Ev. and To. were known to have large uptakes in various metastases, the latter especially having total metastatic tissue which could be equivalent to three or four normal thyroid glands in the rate of turnover of iodine; this is reflected in the high excretion of organic iodine, the highest in fact found in any group. Where analyses have been done on consecutive days after the dose, on the same patient, there is an increase in the percentage of organic iodine excreted with succeeding days, this probably indicating a greater output of iodine by tissue which is being destroyed by radiation.

The chromatographic evidence for the presence of di-iodotyrosine and 3:5-di-iodo-4-hydroxyphenyl-lactic acid is not conclusive proof of their presence in urine, although identity in four different solvents is strong evidence in its favour. Further evidence could be obtained by adding unlabelled di-iodotyrosine and 3:5-di-iodo-4-hydroxyphenyl-lactic acid to urine, isolating these compounds, and recrystallizing them to a constant specific activity. Assuming these identities to be valid, it is pertinent to inquire the source of these compounds. 3:5-Di-iodophenyl-lactic acid can arise by the metabolism of di-iodotyrosine, as shown originally by Foster & Gutman (1930) and confirmed by Stanbury *et al.* (1956*b*), and hence if di-iodotyrosine is present in the circulation 3:5-di-iodo-4-hydroxyphenyl-lactic acid may well appear in the urine. The di-iodotyrosine found in these experiments could be conceived as arising in one of the following ways: (1) as an artifact during the separation; (2) as an artifact in the kidney or bladder; (3) from circulating di-iodotyrosine; (4) from circulating thyro-

globulin, by breakdown in the kidney; (5) from circulating thyroxine, by breakdown in the kidney.

Possibility (1) is extremely unlikely, since the urine is made alkaline soon after it is passed and relatively enormous quantities of carrier iodide are added, and since radioiodination of tyrosine would depend on the presence of free radioactive iodine the possibility of radioiodination is remote. Furthermore, as is shown in Tables 1 and 2, the amount of organic iodine in the urine, and, to a close approximation, the amount of di-iodotyrosine, is dependent on the amount of functioning thyroid tissue and not on the amount of iodide present. If the di-iodotyrosine were an external artifact the quantity of it should parallel the total activity.

Similar arguments may be applied to possibility (2). Iodination in the kidney or bladder, where urine is usually at an acid pH of 5–6, could conceivably occur, but, again, it would then presumably be proportional to the total activity of the urine. Also the amount of iodide excreted per day (about 100 μg.) is totally inadequate for a double iodination of the daily excretion of tyrosine, and hence if iodination occurred, monoiodotyrosine and not di-iodotyrosine would probably be formed.

Urinary di-iodotyrosine could be derived from free di-iodotyrosine in the plasma as (*a*) free di-iodotyrosine released from the thyroid gland; (*b*) di-iodotyrosine produced by the breakdown of thyroxine in the tissues; (*c*) di-iodotyrosine produced by the breakdown of thyroglobulin in the tissues. (*a*) is unlikely since studies of the thyroid have shown that free di-iodotyrosine is present in only small amounts (Tishkoff, Bennett, Bennett & Miller, 1949; Tong, Taurog & Chaikoff, 1951). Since di-iodotyrosine is rapidly removed from the circulation and excreted, and most of it is de-iodinated before excretion, any di-iodotyrosine in the urine would reflect an amount in the plasma about 10 times this (Tong *et al.* 1954). It is improbable that such amounts could be released by the thyroid.

Little is known about possibility (*b*). Although there is no evidence that thyroxine is metabolized by tissues to give di-iodotyrosine, a consideration of the thyroxine molecule shows that hydrolytic or reductive cleavage at the ether oxygen would result in the formation of di-iodotyrosine. This reaction could only give labelled di-iodotyrosine from thyroxine labelled in the 3 or 5 position. Only if a molecule of alanine were involved in the cleavage of the ether bond could di-iodotyrosine be formed from the distal ring of thyroxine. This would in fact be a reversal of synthesis, and would appear to be rather unlikely. However, Albert *et al.* (1949) found a recovery of 15 % of the urinary activity in the di-iodotyrosine fraction after an oral dose of thyroxine labelled in the 3′ position. This result is difficult to interpret. No positive identification of the di-iodotyrosine was made and the analytical method was crude.

The breakdown of thyroglobulin in the plasma (*c*) is another possible source of di-iodotyrosine in the urine. In all the cases studied where simultaneous plasma and urine analyses were carried out, thyroglobulin was present in the plasma. The distribution of the iodine in thyroglobulin is given by Taurog, Tong & Chaikoff (1950) as monoiodotyrosine 15 %, di-iodotyrosine 30 %, thyroxine > 22 %, and by Roche, Jutisz, Lissitsky & Michel (1951) as monoiodotyrosine 33 %, di-iodotyrosine 33 % and thyroxine 27 %. Although reports of the composition of thyroglobulin differ, it seems that it contains about 33 % of the iodine in the form of di-iodotyrosine. The excretion of the breakdown products of thyroglobulin is unknown, but it seems at least possible that the di-iodotyrosine present in it would be excreted eventually in a manner similar to free di-iodotyrosine. From the work of Stanbury *et al.* (1956*a*) the iodine of monoiodotyrosine in thyroglobulin would not be expected to appear in organic combination in the urine.

It is possible from a knowledge of the di-iodotyrosine content of the urine and of the thyroglobulin content of the plasma, to find very approximately the amount of plasma cleared of thyroglobulin in a day.

Thus for patient Be. the total di-iodotyrosine excreted in one day was 43 μc. If it is assumed that 90 % of the di-iodotyrosine in plasma is degraded to iodide before excretion, and only 10 % appears as di-iodotyrosine or 3:5-di-iodo-4-hydroxyphenyl-lactic acid in the urine (Tong *et al.* 1954), then 43 μc of di-iodotyrosine in the urine represents 430 μc of di-iodotyrosine in the plasma. Assuming that the di-iodotyrosine was derived from thyroglobulin in the plasma, then, since the iodine of di-iodotyrosine is approximately one-third of the iodine of thyroglobulin, 430 μc of di-iodotyrosine is equivalent to 1290 μc of thyroglobulin. An analysis of the plasma on the same day showed that it contained 0·04 μc of thyroglobulin/ml. Therefore the amount of di-iodotyrosine found in the urine would be derived from the thyroglobulin contained in 1290/0·04 ml., or 32 l., of plasma.

Therefore the di-iodotyrosine in the urine represents 32 l. of plasma cleared of thyroglobulin. Other results obtained from other patients were 12 and 18 l. of plasma cleared/day.

From these figures it would seem that thyroglobulin is removed from the circulation rapidly. Brown & Jackson (1954, 1956) injected homologous labelled thyroglobulin into several species and found that with the exception of rabbit thyroglobulin, after 24 hr. only a few per cent. of the activity remained in the plasma, and this result is evidence in favour of the figures obtained from the urinary excretion.

These last three sources of urinary di-iodotyrosine presuppose that free di-iodotyrosine is circulating in the plasma. Simultaneous analysis of plasma has

in no case shown the presence of free di-iodotyrosine, but since the analysis was performed on only 50 μl. of plasma, any di-iodotyrosine present may have been too low in amount for detection, but might have been revealed by extracting larger quantities of material.

Di-iodotyrosine could appear in urine without being in the plasma by the breakdown of thyroxine or thyroglobulin in the kidney tissue with the elimination of the di-iodotyrosine thus formed in the urine. There is, however, no evidence on this point and elucidation must await the results of future work. At the moment it would appear probable that the di-iodotyrosine and 3:5-di-iodo-4-hydroxyphenyl-lactic acid of the urine are produced by the breakdown of thyroglobulin.

SUMMARY

1. The urine of patients receiving large doses of radioactive iodine for treatment of thyroid carcinoma has been investigated.

2. The amount of the iodine present in organic combination has been found to be proportional to the known uptake of radioactive iodine by the thyroid or neoplastic tissue.

3. The organic fraction has been studied by paper chromatography.

4. Two main compounds in the organic fraction have been shown to be chromatographically identical with 3:5-di-iodotyrosine and 3:5-di-iodo-4-hydroxyphenyl-lactic acid.

5. Several compounds of unknown structure have also been shown to be present.

6. The origin of the urinary di-iodotyrosine is discussed. It is considered probable that this is derived from circulating thyroglobulin.

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Preparation and Properties of Highly Purified Diaphorase

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In 1937 an enzyme catalysing the reaction between reduced coenzyme I (Co I-H_2) and methylene blue (or other suitable carriers) was found to be present in the plant and animal kingdoms and was named at the time 'coenzyme factor' or diaphorase by two groups of workers who independently discovered it (Green, Dewan & Leloir, 1937; Dewan & Green, 1937; Adler, Euler & Hellström, 1937; Adler, Euler & Günther, 1938). It was subsequently found widely distributed in animal tissues (Green, Needham & Dewan, 1937; Dewan & Green, 1938; Green & Dewan, 1937; Euler & Hasse, 1938; Euler & Günther, 1938*a*); in yeast (Euler & Günther, 1938*b*; Dewan & Green, 1938); and in plant tissues (Lockhart, 1939).

Straub (1939) reported the isolation of a soluble flavoprotein from heart muscle and showed that the prosthetic group was flavinadenine dinucleotide (FAD). Straub, Corran & Green (1939), on testing the catalytic properties of the enzyme, concluded that this flavoprotein was identical with the 'coenzyme factor'; this was confirmed later by Adler, Euler & Günther (1939). A detailed account of the catalytic properties of the enzyme was subsequently published by Corran, Green & Straub (1939). But no criteria of purity such as electrophoresis or sedimentation were applied and, in fact, surprisingly little is known about the physicochemical properties of the enzyme. Straub's best preparation contained $0{\cdot}54 \pm 0{\cdot}02$ % of riboflavin, a figure on which his molecular weight of 70 000 is based, assuming 1 mole of flavin/mole of protein.

It may be thought strange that a classical flavoprotein such as Straub's diaphorase has been so little investigated. Yet it is a somewhat controversial enzyme. Thus Lockhart & Potter (1941) and Potter (1950) suggested that diaphorase might represent a transformation artifact derived from Co I–cytochrome *c* reductase. Edelhoch, Hayaishi & Teply (1952) and Mahler, Sarkar, Vernon & Alberty (1952) have again brought up this possibility. Slater (1950) proposed that in a heart-muscle preparation diaphorase is associated with an unknown factor to form the so-called Co I–cytochrome *c*-reductase system.

It is true that starting from the same material (pig heart) a true diaphorase (without cytochrome *c*-reductase activity) may be isolated by a different procedure from that used for the preparation of the Co I–cytochrome *c*-reductase. But the relationship between the two enzymes still remains obscure. The recent work of Strittmatter & Velick (1956) and Velick & Strittmatter (1956) on the microsomal cytochrome from liver has brought to light a new feature in the Co I–cytochrome *c*-reductase system. They have shown that the large fraction of Co I–cytochrome *c*-reductase activity of the liver which is contained in the microsome fraction can in part be ascribed to a Co I-H_2-specific dehydrogenase which is completely inactive with cytochrome *c* but catalyses the reduction by Co I-H_2 of the microsomal cytochrome b_5. The latter cytochrome reacts directly with cytochrome *c* thereby completing a Co I-H_2–cytochrome *c*-reductase system.

The work described in this paper was undertaken with the object of obtaining more precise information on the purity and properties of diaphorase. The isolation procedure, described in detail, has been slightly modified from that originally developed by Straub (1939) and also from that published by Mahler (1955).

MATERIALS AND METHODS

Reduced coenzyme I (*Co* I-H_2). The disodium salt from yeast was obtained from the Sigma Chemical Co. (St Louis, Mo., U.S.A.). The stock solution (1 mg./ml.) was prepared in 0·002 M-phosphate buffer, pH 7·2. The solution was stored at 4° but deteriorated fairly rapidly and was not kept longer than a fortnight. Concentration was estimated from the molecular-extinction coefficient $\epsilon = 6{\cdot}22 \times 10^3$ l. mole^{-1} cm.$^{-1}$ (Horecker & Kornberg, 1948).

2:6-*Dichlorophenolindophenol*. A sample from British Drug Houses Ltd. was purified by dissolving it in 0·001 M-phosphate buffer, pH 7·5, and extracting the acid form with ether. The ether extract was washed with water, extracted with 0·001 M-Na_2CO_3 solution and the dye was precipitated from the slightly warm (40°) carbonate solution by adding NaCl solution and allowing to cool. The precipitate was filtered off and washed with dilute NaCl solution. The dye was standardized by titration of the iodine liberated from acidified KI (Rosin, 1954), which gave a purity of 86 %. The optical density at 600 mμ of a sample of 2:6-dichlorophenolindophenol was determined after suitable dilution with phosphate buffer, pH 8, and calculated on the basis of 86 % purity, gave $\epsilon = 21 \times 10^3$ l. mole^{-1} cm.$^{-1}$. A stock solution of the dye (10^{-4} M) was prepared in 0·002 M-phosphate buffer, pH 7·2. The solution deteriorated slowly and was freshly prepared once a fortnight.

Other materials. Alumina C_γ was prepared according to the method of Willstätter & Kraut (1923, 1924).

Apoenzyme of D-amino acid oxidase was prepared from sheep kidney according to the method of Negelein & Brömel (1938–39).

Measurements of enzyme activity. The method developed by Edelhoch *et al.* (1952) and Mahler *et al.* (1952), which makes use of the decrease in optical density at 600 mμ when 2:6-dichlorophenolindophenol is reduced, was used for assaying diaphorase activity, but the amount of reagents used differed slightly. Thus 1·5 ml. of 0·2 M-phosphate buffer, pH 7·2, 0·2 ml. of stock Co I-H_2 (see above), 1 ml. of stock dye solution and 0·2 ml. of water were placed in quartz cells (1 cm. path). The optical density at 600 mμ was determined and the enzyme solution (0·1 ml. appropriately diluted) was added at zero time. The change in optical density was followed at 30 sec. intervals for 3 min. Enzymic activity was determined from the initial velocity, corrected for the blank rate without any enzyme. Unless otherwise stated, all determinations were carried out at room temp. ($18 \pm 2°$). The reaction was usually carried out in evacuated Thunberg tubes with attached optical cells (obtainable from the Thermal Syndicate, Ltd., Wallsend, Northumberland), for it was found that aerobically when all the indophenol had been reduced, slow reoxidation occurred. However, the initial velocity of the enzyme reaction, with a large amount of dye, was found to be the same whether carried out aerobically or anaerobically.

Enzyme unit. One enzyme unit is defined as that amount which causes an initial rate of change of optical density at 600 mμ of 0·01/min. under the above conditions. Protein was estimated by the optical density at 280 mμ according to the method of Warburg & Christian (1939–40). An optical density of 1·0 at this wavelength is found to be approximately equivalent to a protein concentration of 1 mg./ml.

Electrophoresis. This was carried out in the Tiselius electrophoresis apparatus (Perkin–Elmer Corpn., Conn.,

U.S.A.; model 38) at 4°. For most of the preparative runs the 6·6 ml. cell was used, though the 2·2 ml. cell was used in the expt. described in Table 2. Photographs were recorded on rapid process P 200 panchromatic plates (Kodak, Ltd.). Before electrophoresis the protein was dialysed at 0° against buffer for 16–19 hr.

Sedimentation. Sedimentation runs were made in the model E Analytical Spinco Ultracentrifuge at room temp. and at top speed (59 780 rev./min.). The protein was dialysed overnight at room temp. against 0·05 M-phosphate buffer, pH 7·2, containing 0·05 M-NaCl.

Diffusion. The Perkin–Elmer electrophoresis apparatus, with the modified Longsworth scanning technique, was used for the diffusion measurement. The boundaries were brought into view by a clockwork mechanism devised by Dr J. Keilin and Mr B. R. Slater. The experiment was done at 17° on the sample of dialysed diaphorase used in the sedimentation experiments. Photographs were taken at intervals over a period of 3 days. The diffusion coefficient was calculated by the height–area method from enlarged projections of the photographs on graph paper.

Spectrophotometric measurements. With the exception of the measurement of the absorption spectrum of diaphorase (Fig. 5), for which the Unicam photoelectric spectrophotometer was used, all measurements were made in the Beckman Model DU spectrophotometer.

Measurement of the flavin content. The flavin content of the protein was determined on a sample of the enzyme solution used for ultracentrifugal analysis. This had previously been exhaustively dialysed against distilled water and made up to 5 ml. The FAD content was determined on a boiled portion of the solution by means of the D-amino acid oxidase system (Warburg & Christian, 1938). The apoenzyme of the D-amino acid oxidase was calibrated against a standard FAD solution. The sample of FAD used in this calibration had been purified on a cellulose column according to the method of Whitby (1953), and was free from interfering adenylic compounds (see Burton, 1951). Chromatography on paper with the solvent system butanol–acetic acid–water (Crammer, 1948), and with a 5% aq. soln. of $Na_2HPO_4,12H_2O$ (ascending chromatogram; Whitby, 1950), showed that FAD was the only fluorescent flavin nucleotide present in the sample. An independent estimate of the FAD content was made by determination of the optical density at 451 mμ, a value of $\epsilon = 1{\cdot}04 \times 10^4$ l. mole.$^{-1}$ cm.$^{-1}$ being used. In both estimations the FAD content was calculated from the dry wt. of the solution after freeze-drying.

Purification procedure

The modified method described below, up to step VIII, is one developed by Drs M. Dixon and G. Weber in this Department. The procedure for further purification is original.

Step I. Mincing and washing. Twelve pig hearts were freed from fat and connective tissue and passed through an electric meat mincer. The mince was repeatedly washed with 20 vol. of tap water until the washings were no longer red (usually eight washings). The mince was stirred vigorously with a mechanical stirrer for 20 min./wash. The mince was then squeezed through calico cloth.

Step II. Preparation of homogenate. The mince was homogenized with 0·02 M-Na_2HPO_4 soln. in a Waring Blendor (200 g. of mince plus 300 ml. of phosphate) for 4·5–5 min. To the homogenate a volume of water equal to the volume of phosphate used was added and the extract spun in an International serum centrifuge for 30 min. at 2000 rev./min. The precipitate was discarded.

Step III. Acidification to pH 4·6. To each l. of supernatant 25 ml. of M-acetate buffer, pH 4·6, was added and the solution left overnight in the cold room. The precipitate was centrifuged (International serum centrifuge for 20 min.) and the supernatant discarded.

Step IV. First heating. The precipitate was suspended in water (1 l./kg. of original washed mince used) and the suspension made as fine as possible by mechanical stirring. Ammonium sulphate to 1% was added. The solution was now quickly heated to 38–40°, kept at this temp. for 5 min., and then rapidly cooled and spun in a refrigerated centrifuge for 30 min. (2000 rev./min.). The precipitate was discarded.

Step V. Treatment with alumina C_γ. The yellow fluorescent supernatant was adjusted, if necessary, to pH 6–6·5 with N-acetic acid (usually the pH needed no adjustment). Alumina solution (65 ml.; dry wt. 18–19 mg./ml.), was added. After 30 min. in the cold the solution was centrifuged. The supernatant was treated with a further quantity of gel to adsorb any remaining diaphorase and the centrifuging repeated. The two lots of alumina were combined and the adsorbed enzyme was eluted with successive lots of 50 ml. each of 0·2 M-Na_2HPO_4 soln., until the eluate ceased to be yellow (usually 4 or 5 lots). The combined eluates were dialysed overnight against two changes of distilled water (15 l. each).

Step VI. Second heating and ammonium sulphate fractionation. The dialysed solution (usually 500 ml.) was made 1% with respect to $(NH_4)_2SO_4$. The solution was heated to 60° by a water bath and kept at this temp. for 5 min. The solution was cooled, centrifuged and the precipitate discarded. The supernatant was then brought up to 0·45 saturation with $(NH_4)_2SO_4$, allowed to stand in the cold for 1 hr. and spun in the Serval High-Speed centrifuge. The precipitate was discarded. The supernatant was then brought up to 0·8 saturation with $(NH_4)_2SO_4$, allowed to stand for 1 hr. in the cold, and again centrifuged. The precipitate was dissolved in about 20 ml. of water.

Step VII. Dialysis. The solution was then dialysed against distilled water until a precipitate appeared. This was spun off and the dialysis continued until further precipitation occurred.

Step VIII. Third heating and second ammonium sulphate fractionation. The precipitate was dissolved by the slow addition of aq. 0·1% (w/v) NH_3 soln. (pH 7–7·4). The solution was now heated to 62° for 5 min. After cooling, the solution was brought up to 0·55 saturation with $(NH_4)_2SO_4$. The precipitate was discarded after centrifuging. The supernatant was then brought up to 0·65 saturation. The diaphorase appeared as a yellow precipitate. It can be stored at this stage in the refrigerator. Alternatively, the $(NH_4)_2SO_4$ precipitate may be centrifuged off, dissolved in dilute phosphate buffer, pH 7–7·5, and stored at −15°. Step VIII corresponds essentially to the last stages of Straub's method of preparation. The yield of enzyme obtained is about 50–90 mg./kg. of original washed mince.

The instability of the enzyme during the initial stages presents a problem, especially during the phosphate extraction just before acidification. When step IV has been started it is advisable to work as rapidly as possible to step VII. Enzyme activity was determined at each stage of the preparation. Table 1 is representative of a typical purification procedure.

Table 1. *Summary of diaphorase purification*

Procedure	Volume (ml.)	Activity (units/ml.)	Protein (mg./ml.)	Purity (units/mg. of protein)	Purification factor	10^{-3} × Total units	Yield (%)
Washed pig-heart mince (2000 g.) extracted with 0·02 M-phosphate	4000	600	44	13·6	—	2400	100
Ppt. obtained after acidification resuspended in water	2000	1100	91	12·0	0·8	2200	91
Suspension heated to 38–40° for 5 min. Supernatant after centrifuging	1600	600	6·2	97·0	7	960	40
Alumina eluate after dialysis	700	680	4·5	151	11	476	20
Ppt. from 0·8 sat. $(NH_4)_2SO_4$, redissolved in water	35	7000	13·6	514	37	245	10
Final 0·65 sat. $(NH_4)_2SO_4$ ppt., redissolved in water	7·8	8100	9·5	852	63	63	2·6

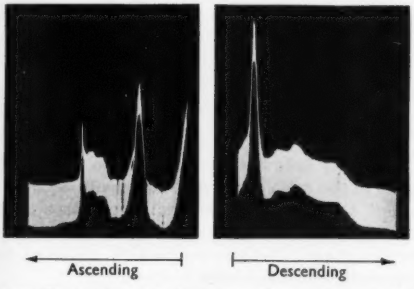

Fig. 1. Electrophoretic diagrams of ascending and descending boundaries of a solution of diaphorase at step VIII in 0·05 M-tris buffer, pH 8·5, 95 min. after beginning of migration.

RESULTS

Purity of preparation

Different diaphorase preparations (step VIII) were examined in the Tiselius electrophoresis apparatus. Electrophoresis was carried out in 0·05 M-2-amino-2-hydroxymethylpropane-1:3-diol (tris) buffer, pH 8·5. (In later experiments the ionic strength of the buffer was increased by the addition of 0·05 M-NaCl.) In all cases a typical pattern was obtained. A similar pattern was obtained in one case with a preparation made exactly according to the procedure of Straub. Thus within 10 min. of commencing the electrophoresis there was a rapid splitting into several components (see Fig. 1). It will be seen that in addition to a major component there are at least five minor components. The flavin content (as riboflavin phosphate) of diaphorase is given by Straub (1939) as 0·66 % (0·54 ± 0·02 % riboflavin). If one assumes this value to be that of pure diaphorase then on the basis of the flavin content, at the step VIII, different diaphorase preparations were 50–90 % pure.

Further purification of diaphorase

It was observed that if the electrophoresis of the impure diaphorase (Fig. 1) was continued for a long time, the aggregate of faster-moving components migrated out of the field of view leaving behind the major component, which had a much lower mobility at this pH. Isolation of the slow component, by removal of the solution from the electrophoresis cell with a syringe and determination of the specific activity, showed that this component represented the diaphorase. Use was then made of this observation for the final purification of diaphorase.

Electrophoresis. The $(NH_4)_2SO_4$ precipitate obtained in step VIII was centrifuged and dissolved in the minimum of water. The protein solution was then dialysed in the cold against distilled water until a precipitate appeared (this precipitate was colourless and was spun off and discarded). The solution was diluted so that the protein concentration was approx. 1–2 %. Dialysis was then carried out against 0·05 M-tris buffer, pH 8·5, containing 0·05 M-NaCl for 16–24 hr. in the cold. Electrophoresis was then carried out in the Tiselius apparatus, with a potential of 100 v and a current of 11 mA. The duration of the electrophoresis varied from run to run but the usual time was 8–9 hr. After each run the descending limb was isolated from the remainder of the cell and the contents were removed. The contents from several runs were combined.

Ammonium sulphate fractionation. The combined fractions, still in 0·05 M-tris, pH 8·5, were treated with $(NH_4)_2SO_4$ up to 0·3 saturation. Some inert protein was precipitated and was removed by centrifuging. The diaphorase was precipitated by bringing the supernatant up to 0·65 saturation with $(NH_4)_2SO_4$.

Table 2 is a summary of the purification procedure. The recovery of diaphorase and its purity (more especially with the purer fractions) can be

Table 2. *Summary of further purification of diaphorase*

For details see Materials and Methods.

Procedure	Volume (ml.)	10^{-3}Activity (units/ml.)	Protein (mg./ml.)	Purity (units/mg. of protein)	Purification factor	10^{-3} × Total units	Yield (%)
Solution before electrophoresis	2·2	20·25	14·7	1730	—	44·5	100
Descending-limb fraction after electrophoresis for 8 hr.	0·8	23·0	6·3	3600	2	18·0	40
Fraction remaining after isolation of descending limb	1·5	16·0	7·7	2616	—	24·0	—
Descending-limb fraction after $(NH_4)_2SO_4$ fractionation and dialysis	2·7	3·5	0·69	5000	2·8	10·0	22

Table 3. *Decrease in* $E_{280\ m\mu}/E_{451\ m\mu}$ *with purification of diaphorase*

Values of E refer to 1 cm. thickness of solutions.

Fraction	$E_{280\ m\mu}$	$E_{451\ m\mu}$	Ratio $E_{280\ m\mu}/E_{451\ m\mu}$
Solution after step VIII, before electrophoresis	9·79	0·95	10·3
Descending limb after electrophoresis	6·3	0·84	7·5
Remainder of solution after electrophoresis	7·7	0·85	9·0
Descending limb after $(NH_4)_2SO_4$ fractionation	6·9	1·15	6·0

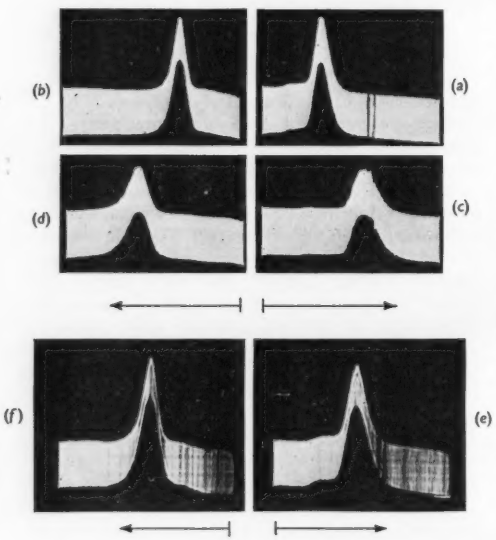

Fig. 2. Electrophoretic diagrams of ascending and descending boundaries of a solution of diaphorase (about 1%) after further purification. (*a*) and (*b*), Descending and ascending limbs respectively, 221 min. after migration in 0·05M-phosphate–0·05M-NaCl, pH 6·5; (*c*) and (*d*), descending and ascending limbs respectively of the same solution after 405 min. migration; (*e*) and (*f*), descending and ascending limbs respectively after 420 min. migration in 0·05M-phosphate–0·05M-NaCl, pH 7·2.

conveniently followed during isolation by determination of the ratio of the optical density at 280 mμ to that at 450 mμ. Table 3 is an example of the method applied to a preparation from the electrophoresis stage. The purest fraction obtained was subjected to physical tests.

Physicochemical properties of the purified enzyme

Electrophoresis. To date there is no published work on the electrophoresis of diaphorase. The fraction obtained by electrophoresis followed by $(NH_4)_2SO_4$ fractionation was examined in the Tiselius apparatus at different pH values.

At pH 7·2 (0·05M-phosphate–0·05M-NaCl) the protein appeared to be homogeneous after electrophoresis for up to 3 hr. When the process was continued for a longer time, however, a small amount of slower-moving component could be detected. At this pH the main component had a mobility of $\mu = 2{\cdot}43 \times 10^{-5}$ cm.^{2} sec.$^{-1}$ v^{-1} (see Fig. 2*e*, *f*).

At pH 6·5 (0·05M-phosphate–0·05M-NaCl) the same picture was obtained, as shown in Fig. 2*a*–*d*. The mobility of the main component was

$$\mu = 2{\cdot}2 \times 10^{-5}\ \text{cm.}^2\ \text{sec.}^{-1}\,\text{v}^{-1}.$$

At both pH values the direction of migration was anodic. In another experiment, carried out in 0·05M-acetate (containing 0·05M-NaCl), pH 5·5, no movement of the boundary was observed after electrophoresis for 5 hr. In acetate, pH 4·89, there appeared to be a very slight movement towards the cathode after electrophoresis for the same period of time. A large amount of precipitation occurred at this pH and the experiment was abandoned. No

electrophoresis experiments were carried out at lower pH values owing to the fact that some irreversible splitting off of the flavin occurred. At pH 8·5, with the same buffer used in the preparative procedure, there was a single component in the ascending boundary but the descending boundary showed the presence of a slower component and the major component itself was somewhat asymmetrical. The mobility was not determined at this pH. With this buffer system distinct boundary anomalies were apparent; these were not observed in veronal buffer, pH 8·5.

Sedimentation. The sedimentation coefficient $S_{20,w}$ was determined at different protein concentrations. $S_{20,w}$ was found to vary slightly with concentration (Fig. 3). A value

$$S_{20,w} = 5{\cdot}38 \times 10^{-13} \text{ cm.}^2\text{/sec.}$$

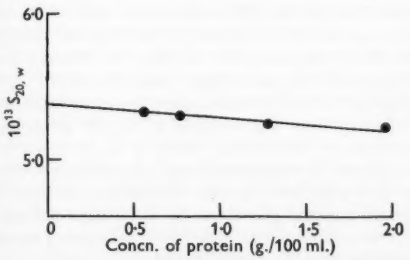

Fig. 3. Relationship between concentration of diaphorase and its sedimentation coefficient in 0·05 M-phosphate–0·05 M-NaCl, pH 7·2.

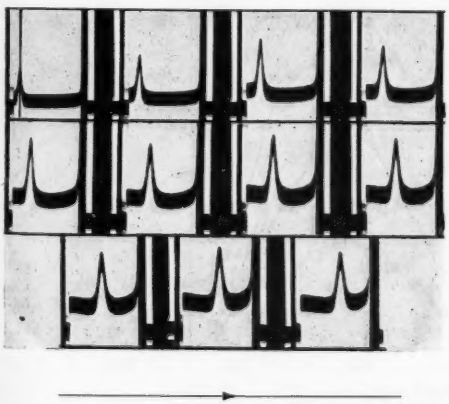

Fig. 4. Sedimentation-boundary diagrams of a 1·2% solution of diaphorase in 0·05 M-phosphate–0·05 M-NaCl, pH 7·2. Speed, 59 780 rev./min. Mean temp. of rotor, 19°. First exposure 16 min. after reaching top speed. Interval between exposures is 8 min.

was obtained by extrapolation to zero protein concentration. In all cases the sedimentation constant was calculated assuming a partial specific volume for the solute of 0·73. The diaphorase in all these experiments showed one component only, and the peak remained symmetrical throughout (see Fig. 4).

Diffusion constant. One diffusion measurement was carried out on a 0·5% solution of diaphorase which had previously dialysed overnight at room temperature against the same solvent system as was used in the sedimentation experiments. A corrected value of $D_{20,w} = 6{\cdot}08 \times 10^{-7}$ cm.²/sec. was obtained.

Molecular weight. The molecular weight has been calculated by using the Svedberg equation (see Svedberg & Pedersen, 1940)

$$M = \boldsymbol{R}Ts/D(1-V_\rho).$$

Combining the extrapolated value for $S_{20,w}$ of $5{\cdot}38 \times 10^{-13}$ cm.²/sec. with the diffusion coefficient $D = 6{\cdot}08 \times 10^{-7}$ cm.²/sec. and an assumed partial specific volume of $V = 0{\cdot}73$, a molecular weight of 81 000 is obtained. Allowing for the assumptions made in this calculation, there is fairly good agreement with the value of 70 000 found by Straub (1939), on the basis of the flavin content and assuming one flavin group per molecule of protein, and that of 72 000 ± 4900 found by Weber (1951), using the polarization of fluorescence technique. The sedimentation experiments now show that there is only one molecule of flavin per mole of protein, a fact that has hitherto been assumed.

Flavin content. The solution of diaphorase of highest purity, containing 0·859 mg./ml., was found to contain 0·01006 mg. of FAD/ml. by the D-amino acid oxidase test, and 0·00998 mg./ml. spectrophotometrically. This corresponds to a FAD content of 1·17 and 1·16% respectively (0·56% riboflavin). The minimum molecular weight, based upon the flavin content, is $786 \times 100/1{\cdot}165 = 67\,000$. On the basis of this flavin content, Straub's preparation would have been about 98% pure.

Absorption spectrum. The absorption spectrum of the purest preparation is similar to that published by Straub (1939), in that there are maxima at 274, 359 and 451 mμ, and minima at 397 and 315 mμ (see Fig. 5). But the ratios of the light absorption at the maxima are somewhat different. The ratio of light absorption at 274 mμ to that at 451 mμ obtained in the present study was 6·72. Comparison with the figure of 7·33 observed for this ratio by Straub indicates that some colourless impurities absorbing in the region 270–280 mμ in Straub's original preparation have been removed in the present preparation. The ratio of absorption at 359 mμ to that at 451 mμ was 0·97 (Straub found 0·91). Fig. 6 shows the absorption spectra of diaphorase in the oxidized and reduced forms in the

visible region. Curve 1 is the oxidized form of diaphorase. Curve 2 was obtained by treating the same solution of diaphorase with excess of reduced Co I. The large amount of absorption by the reducing agent below 400 mμ precludes any accurate measurement in this region. Addition of Co I-H_2 eliminates the fluorescence of the enzyme, but it will be seen from Fig. 6, curve 2 that a large amount of residual absorption still remains in the region of the flavin peak at 450 mμ. If dithionite is added to the enzyme solution reduced by Co I-H_2 there is a further decrease in absorption in the region 450–500 mμ (see Fig. 6, curve 3). Dithionite alone produces a slightly greater decrease in absorption in the visible region than either Co I-H_2 or Co I-H_2 plus dithionite, as shown in Fig. 6, curve 6.

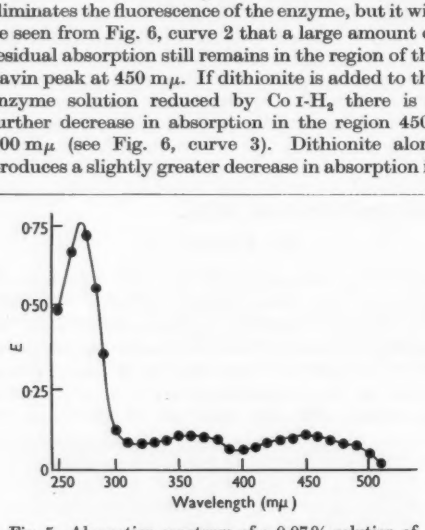

Fig. 5. Absorption spectrum of a 0·07% solution of diaphorase in 0·01 M-phosphate buffer, pH 7·2.

A characteristic feature of the absorption spectrum of diaphorase is the presence of a shoulder at 480 mμ. Straub (1939) reported that examination of a solution of diaphorase under the low-dispersion spectroscope indicated the presence of two bands, one at 451 mμ and another at 480–490 mμ. The difference spectrum, obtained by subtracting curve 2 from curve 1, and plotted in curve 4, clearly indicates the presence of a two-banded spectrum. This phenomenon is not so apparent in the difference spectrum obtained by subtracting curve 3 (Co I-H_2 plus dithionite) from curve 1, as shown in curve 5, which resembles more closely that of the oxidized form (curve 1). Another feature of the absorption curves obtained by reduction of diaphorase with Co I-H_2 or with Co I-H_2 plus dithionite is the increase in general absorption in the region 510–600 mμ. This effect is not observed to the same extent in a solution of the enzyme reduced with dithionite, as shown in curve 6. When a pure solution of FAD was reduced with excess of Co I-H_2 a typical flavin-difference spectrum was obtained, indicating that the anomalies in the absorption spectrum of diaphorase when it is reduced with Co I-H_2 are not apparently an inherent property of the free prosthetic group. Reduction of FAD by Co I-H_2 was brought about by allowing solutions of the two substances to react until no further change in optical density was observed.

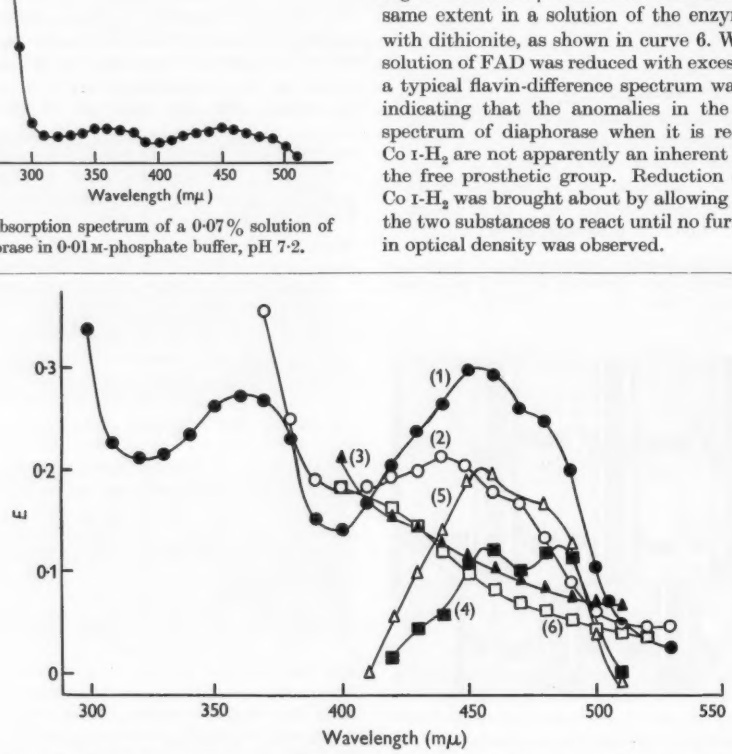

Fig. 6. Absorption spectra of the oxidized and reduced forms of a 0·2% solution of diaphorase. Curve 1 (●), oxidized form; curve 2 (○), diaphorase reduced by excess of Co I-H_2; curve 3 (▲), after reduction with Co I-H_2 plus dithionite; curve 4 (■), difference spectrum obtained by subtracting curve 2 from curve 1; curve 5 (△), difference spectrum between that of oxidized diaphorase and diaphorase reduced with Co I-H_2 plus dithionite; curve 6 (□), enzyme reduced with dithionite alone.

FAD was also reduced with dithionite. The results are shown in Fig. 7. Curve 1 is oxidized FAD. After treatment of the oxidized solution for 120 hr. with excess of Co I-H_2, curve 2 was obtained. Curve 3 represents the difference spectrum between oxidized and reduced forms. Curve 4 is FAD reduced by dithionite, curve 5 the difference spectrum. From these curves it is clear that with both Co I-H_2 and dithionite reduction, typical flavin-difference spectra are obtained. Nor does reduction of FAD by Co I-H_2 give rise to an appreciable increase in general absorption above 500 mμ. Beinert (1956*a*) has studied the spectra of flavins over the wavelength range 230–1300 mμ. By recording families of curves during progressive oxidation of the reduced to the oxidized forms at various conditions of pH and concentration, he has obtained evidence for the existence of semiquinoid intermediates in both their monomeric and dimeric forms. In the spectra of FAD shown in Fig. 5 the conditions are such that these intermediates cannot be detected.

Combination of FAD with the apoenzyme to form diaphorase has resulted in a 15 mμ shift toward shorter wavelengths of the characteristic flavin peak at 375 mμ, whereas the peak at 450 mμ is unaffected. The ratios of the light absorption at the two maxima to the minimum at 397 mμ in the visible region with diaphorase varies quite markedly from the corresponding values for FAD. The ratio $E_{375\ m\mu}:E_{405\ m\mu}$ for FAD is about 1·5; the value of $E_{359\ m\mu}:E_{397\ m\mu}$ for diaphorase is 2·0. Diaphorase gives a value of 2·2 for $E_{451\ m\mu}:E_{397\ m\mu}$ (FAD gives a ratio $E_{450\ m\mu}:E_{405\ m\mu}$ of 1·8). It is doubtful whether these differences can be explained entirely on the basis of the flavin to protein linkage. The possibility of the presence of a trace of a haem-protein impurity cannot be entirely excluded. Haem proteins have a Soret band in the region 400–430 mμ, with very high extinction coefficients in both the oxidized and reduced forms. Contamination of diaphorase with a haem protein, in an amount not detectable electrophoretically, could possibly be observed spectrophotometrically. The fact that when FAD is reduced by dithionite more than half the absorption is abolished in the region 400–430 mμ (see Fig. 7, curve 4), whereas reduction of diaphorase by dithionite (see Fig. 6, curve 6) has resulted in an increase in absorption from 400 mμ to about 410 mμ, and considerable absorption still remains in the region from 410 mμ to about 435 mμ, might be explained on the basis of the presence of a haem impurity.

DISCUSSION

Direct comparison of diaphorase obtained in the present study with that originally isolated by Straub in 1939 is difficult as he has not published sufficient physicochemical information. Methods for determining the homogeneity of proteins at that time were relatively undeveloped and the degree of purity of diaphorase remained an open question. The present work has shown that Straub's method of preparing diaphorase gives a product which, when examined by electrophoresis and in the ultracentrifuge, is not pure. A method for further purification of diaphorase has been developed and

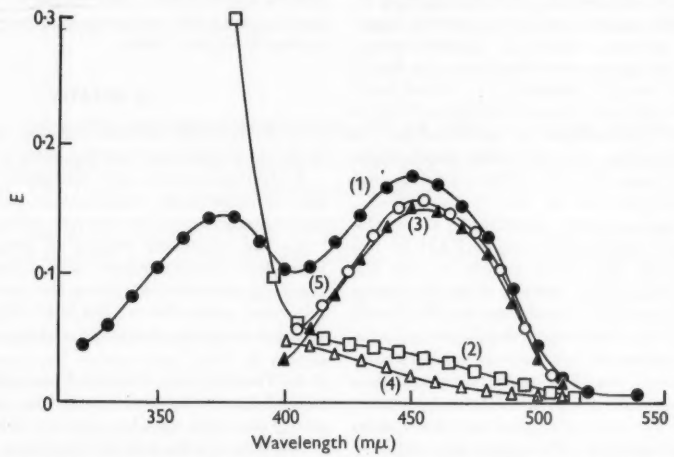

Fig. 7. Absorption spectrum of a $1·5 \times 10^{-5}$ M-solution of FAD. Curve 1 (●), oxidized form; curve 2 (□), reduced with excess of Co I-H_2, after 120 hr.; curve 3 (▲), difference spectrum, i.e. curve 2 subtracted from curve 1; curve 4 (△), FAD reduced with dithionite; curve 5 (○), difference spectrum, i.e. curve 4 subtracted from curve 1.

the enzyme has been obtained at least 95 % pure, as judged by electrophoresis in the Tiselius apparatus and sedimentation in the ultracentrifuge.

From the flavin content of the enzyme Straub (1939) determined the molecular weight of diaphorase and found a value of 70 000, assuming 1 mole of flavin/mole of protein. This assumption is not justified in the absence of experimental evidence; it has been shown that some flavoproteins contain more than one flavin group/molecule. Keilin & Hartree (1948, 1952) have shown that glucose oxidase has two prosthetic groups per molecule of 149 000, while more recently Theorell & Åkeson (1956) have found that the 'old yellow enzyme' has a minimum molecular weight of 52 000 on the basis of the flavin mononucleotide content, but a mol.wt. of 100 000–105 000 as determined by sedimentation. There are thus two flavin mononucleotide groups/molecule of protein.

The absorption spectrum of diaphorase is unusual in that there is an absorption band at about 485 mμ in addition to the usual flavin peak at 450 mμ. A similar band at 490 mμ is observed in the absorption spectrum of L-amino acid oxidase of snake venom, purified by Singer & Kearney (1950). It may be of significance that when Co I-H_2 is added to a solution of diaphorase there is not only a decrease in absorption in the flavin-peak region at 450 mμ but a significant decrease in absorption in the region of the other band at about 485 mμ, as shown by Fig. 6, curve 4, which is the difference spectrum between oxidized diaphorase and diaphorase reduced with Co I-H_2. The significance of this band at 485 mμ is unknown. For lack of any other satisfactory explanation the following may be mentioned. Examination of the absorption spectrum of FAD reveals a slight but definite asymmetry toward the longer wavelengths in the flavin peak at 450 mμ, which is manifest as a broad band from about 440–500 mμ when a solution of FAD is examined with a low-dispersion spectroscope. A solution of diaphorase, on the other hand, when examined in this way shows two distinct bands, one at 450 mμ and another at about 485–490 mμ. Although it has not been possible to show the existence of two separate bands in FAD in the spectrophotometer, the broad nature of the absorption band from 450 to 500 mμ might be due to the presence of two bands. Combination of FAD with the protein to form diaphorase may be such that a more distinct separation of the two bands occurs.

The observation that if Co I-H_2 is added to a solution of diaphorase only about half the absorption in the region at 450 mμ is abolished, and there is an increase in absorption in the region 510–600 mμ, is of interest although it is a phenomenon not entirely new to flavoproteins when reduced by their substrates. Thus Dolin (1956) observed a similar phenomenon in the absorption spectrum of Co I-H_2 peroxidase (a flavoprotein peroxidase) when reduced with Co I-H_2 and dithionite. Dolin interpreted this as a combination of Co I-H_2 with the enzyme to form an enzyme–substrate complex. Beinert & Crane (1956) report the same phenomenon with the three fatty acyl coenzyme A dehydrogenases. The same effect is not observed with a solution of diaphorase reduced by dithionite and it is tempting to interpret this phenomenon as a combination of Co I-H_2 with the enzyme to form an enzyme–substrate complex, as suggested by Dolin (1956) and Beinert & Crane (1956). Beinert (1956*b*), with another flavoprotein, has now shown that this is not the case. By means of a rapid-scanning spectrophotometer and by using a solution of that fatty acyl coenzyme A dehydrogenase which is most active with the C_8–C_{12} derivatives, he showed that this phenomenon is due to the formation of an intermediate oxidation stage. The intermediate is unstable when produced by addition of dithionite in the absence of substrate. The results obtained when diaphorase is reduced by substrate or by dithionite would seem to support Beinert's view.

The present work has not clarified the relationship between diaphorase and cytochrome *c* reductase of pig heart, but it is hoped that this study of some of the properties of diaphorase may be useful in the elucidation of the problem at a future date. Highly purified diaphorase, as now obtained, is completely inactive with cytochrome *c* as electron acceptor, nor has it been possible to convert diaphorase into a cytochrome *c* reductase by the addition of either Fe^{2+} or Fe^{3+} ions. In the absence of any added factors diaphorase is also found to be completely inactive with the cytochrome b_5 isolated by Strittmatter & Velick (1956).

SUMMARY

1. A modified method for the preparation of Straub's diaphorase from pig heart is described.

2. Sedimentation and diffusion measurements and electrophoretic examination have established the homogeneity of the enzyme preparation.

3. The molecular weight of diaphorase calculated from sedimentation and diffusion measurements is about 80 000. From the flavin content the minimum molecular weight is 67 000, which shows that the molecule of enzyme contains one prosthetic group.

4. The addition of reduced coenzyme I to a solution of diaphorase under anaerobic conditions only partly abolishes the absorption at 450 mμ and there is an increase in absorption above 510 mμ. The difference spectrum shows the presence of a further peak with maximum absorption at 485 mμ, in addition to the usual flavin peak at 450 mμ.

I wish to thank Dr M. Dixon, F.R.S., for suggesting the problem and for many valuable suggestions throughout the course of the work. I am grateful to Dr E. Webb for his help and advice. I am indebted to Mr B. Boon for carrying out the ultracentrifuge determinations and to Mr B. R. Slater for his help and co-operation with the electrophoresis experiments and for carrying out the diffusion measurement. I also wish to thank Mr D. Gardiner for much assistance during the preparation of diaphorase. This paper is published with the permission of the South African Council for Scientific and Industrial Research, to whom I am grateful for the award of a Research Bursary.

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Studies in the Biochemistry of Micro-Organisms

102. QUADRILINEATIN (1:2-DIFORMYL-5-HYDROXY-3-METHOXY-4-METHYLBENZENE), A METABOLIC PRODUCT OF *ASPERGILLUS QUADRILINEATUS* THOM & RAPER*

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(*Received* 18 *March* 1957)

Aspergillus quadrilineatus Thom & Raper, a species in the *A. nidulans* group, has been shown by Howard & Raistrick (1955) to produce in the perithecia a crystalline polyhydroxyanthraquinone pigment which has been named asperthecin. By degradative methods Neelakantan, Pocker & Raistrick (1957) have demonstrated that asperthecin is either 3:4:5:6:7-pentahydroxy-2-hydroxymethylanthraquinone or 3:4:5:7:8-pentahydroxy-2-hydroxymethylanthraquinone.

Howard & Raistrick (1955) noted the almost immediate production of a copious, heavy, dark-orange precipitate on the addition of Brady's reagent (2:4-dinitrophenylhydrazine in dilute hydrochloric acid) to the culture solution from strain S.M. 297 of the same organism. They recrystallized

* Part 101: Neelakantan, Pocker & Raistrick (1957).

the precipitate from acetone and obtained orange-red needles of m.p. 235° (decomp.) but did not examine them further. The investigation of the product responsible for this reaction forms the subject of the present communication. A description of the earlier stages of this work was incorporated in a Ph.D. thesis of London University (Lahoz-Oliver, 1955).

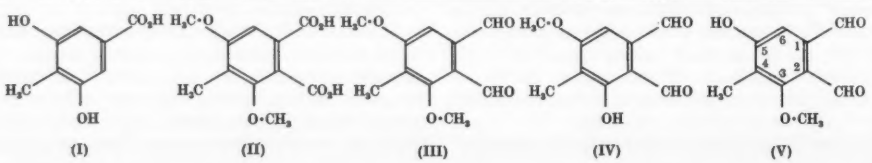
The separation of the product from the culture fluid was most readily accomplished by adsorption on active charcoal. The product could be extracted by means of ether from the charcoal after drying. The solid residue from the ether extract was recrystallized from benzene, which afforded a pure colourless crystalline product which has been named quadrilineatin.

Analysis of quadrilineatin, m.p. 172°, for elementary composition, methoxyl and *C*-methyl groups suggested the empirical formula, $C_{10}H_{10}O_4$, containing one methoxyl and one *C*-methyl group. The substance is neutral in reaction and possesses one hydroxyl group which can be acetylated or methylated. It affords a mono- and a bis-2:4-dinitrophenylhydrazone and a dioxime, indicating the presence of two reactive carbonyl groups. Oxidation with Doeuvre's (1927) reagent (alkaline mercuric iodide in potassium iodide solution) affords a crystalline monobasic acid, $C_{10}H_{10}O_5$, which still gives carbonyl reactions, indicating the presence of at least one formyl group in quadrilineatin. Oxidation of quadrilineatin with alkaline hydrogen peroxide gives in small yield a monobasic acid, isomeric but not identical with that obtained by the Doeuvre reaction. This acid also gives carbonyl reactions. This points to the presence of a second formyl group in quadrilineatin. The functions of all the oxygen atoms are thus accounted for; they are present, one as phenolic hydroxyl, one as methoxyl and two as formyl groups. Since a *C*-methyl group was detected and since benzene derivatives are readily obtained from quadrilineatin we may write the formula as $C_6H(CH_3, OH, OCH_3, 2CHO)$.

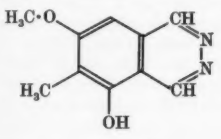
Considerable light was thrown on the arrangement of the substituents in the benzene nucleus by oxidation of the methyl ether of quadrilineatin with cold potassium permanganate. This produced a dibasic acid $C_{11}H_{12}O_6$ [represented as $C_6H(CH_3, 2\,O{\cdot}CH_3, 2\,CO_2H)$], which was an *o*-phthalic acid since it readily formed an anhydride. This phthalic acid on demethylation with hydrobromic acid lost also a carboxyl group and yielded 3:5-dihydroxy-*p*-toluic acid (I) identical with an authentic specimen. Owing to the symmetry of the molecule the carboxyl group eliminated must have occupied the 2-position, the phthalic acid precursor having the structure 3:5-dimethoxy-4-methylphthalic acid (II). For further confirmation the acid of structure (II) was synthesized and found to give no depression in m.p. when mixed with the phthalic acid obtained by permanganate oxidation of *O*-methylquadrilineatin. The anhydrides of these two acids were similarly compared and found to be identical. The two carboxyl groups of the phthalic acid must have arisen from the oxidation of the two formyl groups. Quadrilineatin methyl ether must therefore be an *o*-dialdehyde of structure (III).

On the evidence so far adduced, quadrilineatin must have structure (IV) or (V), depending on which of the two methoxyl groups present in the methyl ether (III) is found as free hydroxyl in quadrilineatin. An immediate clue to help decide this point is the fact that quadrilineatin gives no colour reaction with ferric chloride. It is inconceivable that a phenolic hydroxyl group in the *ortho* position to a formyl group should not give a colour. Further, neither of the two isomeric oxidation acids $C_{10}H_{10}O_5$ obtained, one by Doeuvre oxidation and the other by alkaline hydrogen peroxide oxidation, gives a ferric chloride reaction, yet one of these must have a carboxyl group in the 2-position (CHO at 1), adjacent to the 3-position, occupied by OH or $O{\cdot}CH_3$. If OH were the substituent at C-3 a strong ferric chloride colour would be expected (cf. salicylic acid). Hence the substituent at C-3 must be methoxyl, C-5 being reserved for the hydroxyl group. Quadrilineatin is therefore 1:2-diformyl-5-hydroxy-3-methoxy-4-methylbenzene (V).

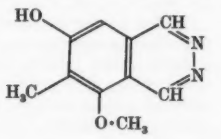
Confirmatory evidence was obtained by treating quadrilineatin with hydrazine or semicarbazide. These reagents afford a phthalazine $C_{10}H_{10}N_2O_2$ crystallizing ($+0{\cdot}2H_2O$ even after drying at 100°) in slightly yellow needles of m.p. 226–228° and giving virtually no colour reaction with ethanolic ferric chloride (which incidentally supports the formulation of quadrilineatin as a phthalaldehyde). The phthalazine obtained should have the structure 5-hydroxy-7-methoxy-6-methylphthalazine (VI) or 7-hydroxy-5-methoxy-6-methylphthalazine (VII) according as which of the two possible positions is occupied by the methoxyl group.



In the study of cyclopaldic acid by Birkinshaw, Raistrick, Ross & Stickings (1952) the phthalazine of established structure (VI) was obtained by the action of hydrazine on the acid, one molecule of carbon dioxide being lost in the process. This phthalazine had m.p. 260–262° (decomp.) and gave an orange–red colour with ethanolic ferric chloride. It is obviously different from the isomeric product obtained from quadrilineatin, which must therefore



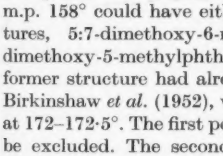
(VI)



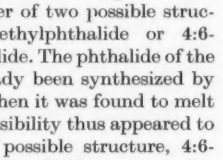
(VII)

have the structure (VII), confirming structure (V) for quadrilineatin. When these two phthalazines obtained from different sources were subjected to demethylation by hydrobromic acid, the products (isolated as the hydrobromide + $\frac{1}{2}C_2H_5 \cdot OH$ in each case) both melted at 238–240° (decomp.) and were identical since they showed no depression on mixing. This observation again confirms the correctness of the general structure ascribed to quadrilineatin.

When quadrilineatin is warmed with dilute sodium hydroxide it readily isomerizes to a phthalide, an internal Cannizzaro reaction to be expected from an *o*-dialdehyde. In order to determine which of the formyl groups is oxidized and which reduced in the rearranged form, the free hydroxyl group of the phthalide was methylated. The product of m.p. 158° could have either of two possible structures, 5:7-dimethoxy-6-methylphthalide or 4:6-dimethoxy-5-methylphthalide. The phthalide of the former structure had already been synthesized by Birkinshaw *et al.* (1952), when it was found to melt at 172–172·5°. The first possibility thus appeared to be excluded. The second possible structure, 4:6-dimethoxy-5-methylphthalide (VIII), was synthe-



(VIII)

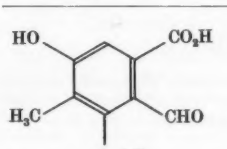


(IX)

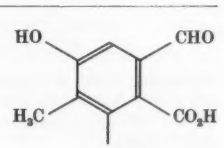
sized by chloromethylation of 3:5-dimethoxy-*p*-toluic acid, followed by hydrolysis and ring closure. It melted at 158° and gave no depression in melting point when mixed with the phthalide obtained as described from quadrilineatin. Therefore the phthalide formed from quadrilineatin under alkaline conditions must have the structure 6-hydroxy-4-methoxy-5-methyl phthalide (IX).

In the earlier work on quadrilineatin (Lahoz-Oliver, 1955) small amounts of a product of higher melting point were frequently obtained from the mother liquors on recrystallization of crude quadrilineatin with toluene or xylene as solvent. This was thought to be a second metabolic product. It has now been shown that this higher-melting product is identical with the phthalide obtained as described above. It must be regarded as an artifact and not as a second metabolite, since when benzene was used as solvent for crystallization and precautions were taken to avoid prolonged heating the phthalide could not be detected. Possibly traces of alkali in the glass of the vessels used, together with the temperature effect, conduced to its formation.

The two isomeric acids obtained by oxidation of one or other of the two formyl groups of quadrilineatin with Doeuvre's reagent and with alkaline hydrogen peroxide respectively must have the constitution (X) or (XI). In order to decide how these



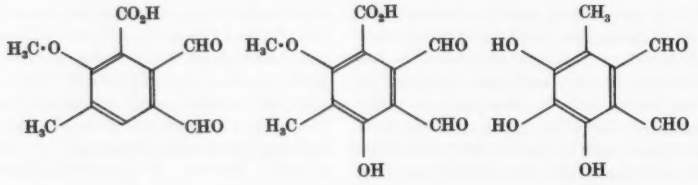
(X)



(XI)

structures should be allocated the acid produced by Doeuvre oxidation was subjected to reduction by zinc dust in methanol containing sulphuric acid. The –CHO group was reduced to $-CH_2 \cdot OH$ and a phthalide was obtained. This was methylated to a dimethoxyphthalide which was found to be identical (no m.p. depression on mixing) with the 5:7-dimethoxy-6-methylphthalide of m.p. 172–172·5° previously synthesized (Birkinshaw *et al.* 1952). This phthalide must have arisen from structure (XI), hence the acid produced by the Doeuvre reaction is 6-formyl-4-hydroxy-2-methoxy-3-methylbenzoic acid. The acid obtained by alkaline hydrogen peroxide oxidation of quadrilineatin must therefore have the alternative structure 2-formyl-5-hydroxy-3-methoxy-4-methyl benzoic acid (X).

Three other phthalaldehyde derivatives have been recorded as fungal metabolites: gladiolic acid (XII) from *Penicillium gladioli* (Brian, Curtis & Hemming, 1948; Grove, 1952; Raistrick & Ross, 1952), cyclopaldic acid (XIII) from *P. cyclopium* (Birkinshaw *et al.* 1952) and flavipin (XIV) from



(XII) (XIII) (XIV)

Aspergillus flavipes and *A. terreus* (Raistrick & Rudman, 1956). Of these cyclopaldic acid (XIII) is most closely related to quadrilineatin as regards type and arrangement of substituents; in fact norquadrilineatin would be identical with decarboxynorcyclopaldic acid.

Quadrilineatin was found to have only feeble antibacterial activity against *Staphylococcus aureus* and *Escherichia coli*. Since the three phthalaldehydes (XII), (XIII) and (XIV) were found to have considerable antifungal activity it became of interest to determine the behaviour of quadrilineatin in this respect. The antifungal activity, determined by Mr G. Smith, is recorded in the Appendix.

EXPERIMENTAL

All melting points are uncorrected. Elementary analyses and acetyl determinations are by Drs Weiler and Strauss, Oxford. Methoxyl determinations are by two of us (P.C. and R.L.-O.).

History of cultures

Five strains of *Aspergillus quadrilineatus* Thom & Raper were examined and compared. These were as follows: A 54a and A 54b were received in June 1942 from Dr K. B. Raper, of the Northern Regional Research Laboratory, Peoria, Illinois, and bore the designations NRRL 521 and NRRL 201 respectively. S.M. 200 and S.M. 297 were isolated from two different samples of Australian soil by Mrs S. Marcus in October 1950 and February 1951 respectively. G.A. 317 was isolated from Indian soil by Mr G. Agosti in August 1954.

The three soil strains were all isolated in this Department and were identified by Mr G. Smith. Since strain S.M. 297 gave the best yield of quadrilineatin (see Table 1) it was employed in the preparation of supplies of this metabolite needed for characterization and determination of molecular structure.

Cultural conditions

The culture medium employed throughout this work was the Raulin–Thom solution containing: glucose, 75 g.; tartaric acid, 4·0 g.; ammonium tartrate, 4·0 g.; $(NH_4)_2HPO_4$, 0·6 g.; $(NH_4)_2SO_4$, 0·25 g.; K_2CO_3, 0·6 g.; $MgCO_3$, 0·4 g.; $FeSO_4,7H_2O$, 0·07 g.; $ZnSO_4,7H_2O$, 0·07 g.; distilled water, 1·5 l. This was distributed in 1 l. conical flasks (350 ml./flask), which were plugged with cotton wool and sterilized by steaming on three successive days. The flasks were inoculated with a suspension in sterile distilled water of spores of the required strain, which had been cultivated on Czapek–Dox agar or wort–agar slopes. The inoculated flasks were incubated at 24° in the dark.

All the strains examined grew well but showed minor differences in appearance and reactions as recorded below.

Strain A 54a. The mycelium was folded, buff-brown in colour, with a deep purplish brown reverse. The pale-straw-coloured culture solution with Brady's reagent gave an immediate turbidity, followed by deposition of a fairly heavy orange–red ppt. It gave a slight brown colour with aqueous $FeCl_3$.

Strain A 54b. The upper surface of the mycelium was greyish brown, the under surface greyish white. The pale-yellow culture solution gave no turbidity or ppt. with Brady's reagent except on standing. There was no colour with $FeCl_3$.

Strain S.M. 200. The mycelium was dark yellowish brown on the upper, deep brown on the under surface. The golden-yellow culture solution gave only a slight orange ppt. with Brady's reagent and no reaction with $FeCl_3$.

Strain S.M. 297. The deeply-folded brown mycelium had a deep purplish brown reverse. The golden-yellow culture solution gave an immediate heavy red ppt. with Brady's reagent and a slight brown colour with $FeCl_3$.

Strain G.A. 317. The heavily folded mycelium was light buff-brown with a deep purplish brown reverse and showed numerous transpiration drops. The golden-yellow culture solution gave an immediate heavy red ppt. with Brady's reagent and no immediate colour with $FeCl_3$; a slight brown colour developed on standing.

The strains employed for the production of asperthecin were S.M. 200 and S.M. 297, as recorded by Howard & Raistrick (1955), who give further details about these two strains.

Harvesting of cultures and isolation of product

The method of harvesting one of the later batches of strain S.M. 297, as evolved when procedure had been standardized, is described below. It represents the typical method of working up and isolation of the product. For strain G.A. 317 a somewhat different procedure had to be adopted. This is also described.

Typical method as applied to strain S.M. 297. After incubation for a period of about 30 days the contents of the batch of flasks was mixed and filtered. The filtrate was stirred with activated charcoal (3 g./l.) for 2 hr.; the charcoal was recovered by filtration and dried *in vacuo* over conc. H_2SO_4. The filtrate no longer gave a ppt. with Brady's reagent. The dried charcoal was then exhaustively extracted with ether in Soxhlet extractors. The combined solid residues (6·65 g.) recovered from the ether were recrystallized once from benzene to yield needles (6·4 g.) of m.p. 169–170°. The tarry residues from the ether extracts and the mother liquor from the crystallization showed no tendency to crystallize and were not further examined. No evidence of a second,

higher-melting product was obtained. By further crystallization from benzene or water and sublimation in a high vacuum the m.p. was raised to a constant value of 172° (with slight decomp.).

Method applied to strain G.A. 317. The culture solution of strain G.A. 317 had given a heavy ppt. with Brady's reagent in the preliminary tests and appeared therefore to be a promising source of quadrilineatin. However, when a batch of ninety-eight flasks of this strain was harvested and the culture solution was treated with charcoal as above, it was found that the ether extract from the charcoal was a tar (12 g.), which showed no sign of crystallization even on prolonged cooling. It was adsorbed from benzene (200 ml.) on acid-washed alumina (200 g.), and the column was eluted successively with benzene (1 l.), benzene–ether (1:1, 1 l.) and ether–methanol (1:1; 500 ml.). The first two eluates were combined and the solution was concentrated to small volume, when crystalline material (3·6 g., m.p. 155–160°) was obtained. Removal of the solvent from the final eluate gave only a deep-brown tar (8 g.), from which no crystalline material could be obtained even by repeated chromatography. Recrystallization of the crystalline solid from benzene gave needles (3·15 g.; m.p. 168–170°) of quadrilineatin.

Properties of quadrilineatin

The pure product forms colourless needles of m.p. 172° (decomp.), and empirical formula $C_{10}H_{10}O_4$, light-absorption max. 250 and 297–300 mμ in ethanol (log ε 4·27, 3·71 respectively). It readily dissolves in hot water but is only sparingly soluble in the cold. It is very soluble in acetone and ether, and fairly so in cold ethanol. It is only slightly soluble in boiling $CHCl_3$ and in boiling light petroleum (b.p. 40–60°). It may be recrystallized from benzene. In aqueous or ethanolic solution no colour reaction is observed with aqueous or ethanolic $FeCl_3$. It is not soluble in a freshly-prepared cold saturated solution of $NaHCO_3$, but is soluble on warming to give a yellow solution. It is readily soluble in aqueous Na_2CO_3 to give a yellow solution and aqueous NaOH to give a yellowish brown solution, decolorized on acidification. It does not reduce Fehling's solution but does reduce ammoniacal $AgNO_3$ soln. It gives a ppt. of Hg when treated with Doeuvre's reagent (aqueous HgI_2 in KI and NaOH). It darkens somewhat on exposure to air. With the Gibbs (1927) reagent in borate buffer (pH 9·2) it gives a greenish blue solution almost immediately. This becomes more blue up to 1 hr., when the colour becomes permanent. It gives no reaction in the following tests for the $H_3C{\cdot}CO$-group: (*a*) iodoform reaction, (*b*) with sodium nitroprusside or (*c*) with *m*-dinitrobenzene in alkaline solution [Found (on a sublimed specimen, m.p. 172°): C, 62·2, 62·2; H, 4·9, 5·35; OMe, 15·8, 15·8; *C*-Me, 5·1; $C_{10}H_{10}O_4$ requires C, 61·8; H, 5·2; (one) OMe, 16·0; (one) *C*-Me, 7·7%].

Antibacterial activity. Quadrilineatin (50 mg.) was dissolved in hot sterile distilled water (15 ml.), cooled quickly and made up to 25 ml. to give a 1:500 dilution, from which further dilutions (two sets) starting at 1:1000 were prepared with sterile heart broth–2% glucose medium. These were inoculated with 24 hr. cultures of *Staphylococcus aureus* N.C.T.C. 6571 and *Escherichia coli* N.C.T.C. 86 respectively, and incubated at 37° for 24 hr. With *Staph. aureus* there was no growth at 1:1000, partial growth at 1:2000, and full growth at 1:4000. With *Esch. coli* complete growth occurred at 1:2000. Quadrilineatin has thus only weak antibacterial activity against the strains tested.

Derivatives of quadrilineatin

Methyl ether. Quadrilineatin (0·08 g.) was dissolved in dry acetone (8 ml.); dry K_2CO_3 (1·6 g.) and dimethyl sulphate (0·8 ml.) were added to the solution. The mixture was refluxed for 2 hr. The excess of K_2CO_3 was removed by filtration and the acetone was distilled off. The residual solid was treated with water. The dark-brown crystalline material was collected and purified by sublimation in high vacuum. Colourless needles of O-*methylquadrilineatin*, m.p. 146–148°, were thus obtained. The product could be recrystallized

Table 1. *Cultural details and yield of product from various strains of* Aspergillus quadrilineatus *grown on Raulin–Thom medium at* 24°

Strain	No. of flasks	Incubation (days)	Residual glucose (%)	Final pH	Yield (g.) and m.p. of crude solid extracted by ether from charcoal	Yield (g.) and m.p. of once-recryst. material	Wt. (g.) of residual non-cryst. material
G.A. 317	98	21	0·60	4·2	3·57* 155–160°	3·15 168–170°	8
A 54a	22	32	0·36	4·6	2·57 168–170°		
S.M. 200	21	32	0·71	4·8	0·41 169–171°	6·2 169–171°	0·64
S.M. 297	22	32	0·42	4·8	3·72 169–171°		
A 54b	25	32	0·42	4·4	Nil	—	—
S.M. 297	100	28	0·46	4·4	6·65 168–171°	6·41 169–170°	0·22
S.M. 297	100	28	0·33	4·6	8·40 168–170°	8·01 169–170°	0·36

* Isolated by chromatography from 12 g. of tar.

from light petroleum, b.p. 80–100° [Found: C, 63·1; H, 5·7; OMe, 30·4. $C_{11}H_{12}O_4$ requires C, 63·4; H, 5·8;(two) OMe, 29·8%].

Acetate. Quadrilineatin (0·4 g.) was heated for 45 min. at 140° (bath temp.) with anhydrous sodium acetate (0·8 g.) and acetic anhydride (2 ml.). Water (20 ml.) was added to the cooled mixture. The oil separating solidified on shaking the mixture. The solid was collected and dried (0·46 g., m.p. 120–132°). Recrystallization from light petroleum (b.p. 80–100°) with addition of decolorizing charcoal afforded irregular plates of *acetylquadrilineatin*, m.p. 140–143° [Found: C, 60·8; H, 5·3; $H_3C{\cdot}CO{\cdot}$, 21·0. $C_{12}H_{12}O_5$ requires C, 61·0; H, 5·1; $H_3C{\cdot}CO{\cdot}$, 18·2%].

Quadrilineatin bis-2:4-dinitrophenylhydrazone. 2:4-Dinitrophenylhydrazine (0·5 g.) was dissolved in warm conc. H_2SO_4 (1 ml.) and the solution was diluted with ethanol (10 ml.). This solution was mixed with a solution of quadrilineatin (0·2 g.) in ethanol (10 ml.). A cherry-red product separated at once, which changed to orange–red in 5 min. The crude crystalline bishydrazone was collected, washed with a little ethanol and dried; it had m.p. 246–247°. After three recrystallizations from butanol the *bis-2:4-dinitrophenylhydrazone* was obtained as orange–red needles of m.p. 262–263° (decomp.) [Found: C, 48·0, 47·6; H, 3·3, 3·1; N, 17·1, 17·8; OMe, 5·7. $C_{22}H_{18}O_{10}N_8$ requires C, 47·7; H, 3·3; N, 20·2; OMe, 5·6%]. A drop of dilute NaOH added to an ethanolic solution of the bishydrazone gave a red colour, indicating that the parent substance was not an α-dicarbonyl compound.

Mono-2:4-dinitrophenylhydrazone of quadrilineatin. This derivative was readily obtained from the culture solution after harvesting. The culture solution (1 l.) from strain S.M. 297 was treated with Brady's reagent [2:4-dinitrophenylhydrazine (0·4%) in 2N-HCl; 500 ml.]. A reddish orange amorphous ppt. was immediately produced. The mixture was kept overnight at 0°. The ppt. was collected and crystallized from nitrobenzene, giving crimson hair-like needles (0·71 g.) of the *mono-2:4-dinitrophenylhydrazone* of quadrilineatin of m.p. 234–235° (decomp.). Further recrystallizations from butanol did not raise the m.p. [Found: C, 51·5; H, 4·5; N, 14·5. $C_{16}H_{14}O_7N_4$ requires C, 51·3; H, 3·8; N, 15·0%]. With strain G.A. 317, the culture solution (100 ml.) was treated with Brady's reagent (200 ml.) and was left for 1 hr. with occasional shaking. The orange–red ppt. formed was collected and dried (m.p. 185–190°). The filtrate was left for 4 days and the brownish red ppt. was collected (0·1 g., m.p. 183–186°). The combined ppts. were crystallized from nitrobenzene, which raised the m.p. to 233–235° (decomp.). By fractional crystallization from butanol and dioxan the crystals were separated into two distinct products: (*a*) the mono-2:4-dinitrophenylhydrazone (0·15 g.), m.p. 235–236° (decomp.), jagged red needles; (*b*) the bis-2:4-dinitrophenylhydrazone (0·03 g.), m.p. 264° (decomp.), orange–red needles. These two products did not depress the m.p. of the mono- and the bis-2:4-dinitrophenylhydrazones respectively previously obtained from strain S.M. 297 culture fluid and from pure quadrilineatin [Found for (*a*): C, 51·2; H, 4·5; N, 14·7; OMe, 8·3. $C_{16}H_{14}O_7N_4$ requires C, 51·3; H, 3·8; N, 15·0; OMe, 8·3%. Found for (*b*): C, 47·7; H, 3·4; N, 20·0; OMe, 6·0. $C_{22}H_{18}O_{10}N_8$ requires C, 47·7; H, 3·3; N, 20·2; OMe, 5·6%].

Quadrilineatin dioxime. A solution of quadrilineatin (0·1 g.) in pyridine (0·15 ml.) mixed with a solution of hydroxylamine hydrochloride (0·1 g.) in ethanol (2 ml.) was refluxed for 3 hr. Water (25 ml.) was added and the solution was evaporated under reduced pressure to half volume. The product, in the form of brownish needles (0·1 g., m.p. 188–192°) was recrystallized from water with addition of charcoal. The *dioxime* was obtained as cream-coloured needles of m.p. 205–206° [Found: C, 53·8, 53·8; H, 5·5, 5·5; N, 12·1, 12·5. $C_{10}H_{12}O_4N_2$ requires C, 53·6; H, 5·4; N, 12·5%].

Oxidation of quadrilineatin

Doeuvre reaction: production of 6-formyl-4-hydroxy-2-methoxy-3-methylbenzoic acid. Quadrilineatin (0·51 g.) was suspended in water (5 ml.) and a solution of HgI_2 (3 g.) and KI (10 g.) in water (20 ml.) was added, followed by N-NaOH (40 ml.). The bright-yellow solution soon began to deposit Hg. It was shaken frequently. After 6 hr. all the crystalline solid had disappeared and the Hg precipitation appeared complete. The solution was filtered through kieselguhr to remove Hg, and the filtrate and washings were titrated to phenolphthalein with N-HCl. The titration value (32·0 ml.) showed that 8·0 ml. of N-acid had been produced in the reaction. (For the conversion

$$R{\cdot}CHO + HgI_2 + H_2O \rightarrow R{\cdot}CO_2H + 2HI + Hg,$$

which requires the production of 3 equiv. of acid, the calc. value is 7·87 ml.) The solution was acidified to Congo red with conc. HCl. On chilling, straw-coloured needles slowly separated and were collected (0·24 g., m.p. 174–176°). Recrystallization from water gave almost colourless needles (0·16 g.) of *6-formyl-4-hydroxy-2-methoxy-3-methylbenzoic acid*, m.p. 176°. The acid was soluble in aqueous $NaHCO_3$ and Na_2CO_3 to give colourless solutions and in cold 2N-NaOH to give a slightly yellow solution. Brady's reagent added to an ethanolic solution of the acid produced an immediate turbidity, followed by deposition of an orange–yellow ppt. With the Gibbs reagent the reaction was similar to that of the parent compound [Found on a specimen dried for 1½ hr. in high vacuum at 70°: C, 55·1; H, 5·1; OMe, 13·85%; equivalent by titration, 215. $C_{10}H_{10}O_5,\frac{1}{2}H_2O$ requires C, 54·75; H, 5·1; OMe, 14·2%; equivalent (monobasic), 219].

2:4-Dinitrophenylhydrazone. The mother liquors from the crystallization of the above acid were treated with an excess of Brady's reagent. After 6 hr. the ppt. was collected and dried [0·11 g., m.p. 263–266° (decomp.)]. Recrystallization from butanol and acetic acid gave microscopic orange-yellow needles (45 mg.) of *6-formyl-4-hydroxy-2-methoxy-3-methylbenzoic acid 2:4-dinitrophenylhydrazone*, m.p. 267–268° (decomp.) [Found (on a specimen dried at 120° in high vacuum); C, 49·2; H, 3·8; N, 13·7; OMe, 8·0. $C_{16}H_{14}O_8N_4$ requires C, 49·2; H, 3·6; N, 14·35; OMe, 7·95%].

Oxidation of quadrilineatin with alkaline hydrogen peroxide: production of 2-formyl-5-hydroxy-3-methoxy-4-methylbenzoic acid. Quadrilineatin (1 g.) was dissolved in 2N-NaOH, and to the deep-yellow solution was added H_2O_2 (3%; 7 ml.) in one portion. The colour of the solution changed quickly to wine-red and the temp. rose to about 40°. When the reaction mixture had cooled to room temp. it was acidified to Congo red with 2N-H_2SO_4, when a little oil separated and was removed. The solution was then treated with a slight excess of $NaHCO_3$ and extracted with ether (4 × ¼ vol.). Removal of the ether left a greenish brown amorphous residue (0·42 g.), which when recrystallized from water afforded needles (0·33 g., m.p. 169–170°) of unchanged quadrilineatin. The aqueous portion was now reacidified and the oil produced was extracted with 4 × ¼ vol.

of ether. The deep-brown tarry residue (0·45 g.) from the ether was subjected to sublimation in a high vacuum at 160–170°. The sublimate (55 mg.) on recrystallization from water formed colourless needles (25 mg.) of *2-formyl-5-hydroxy-3-methoxy-4-methylbenzoic acid*, m.p. 218°. This product gave a deep greenish blue colour with the Gibbs reagent after 5 min.; the colour deepened further up to 1 hr. [Found (on a specimen sublimed in high vacuum): C, 56·5; H, 4·8; OMe, 14·5%; equivalent by titration, 212. $C_{10}H_{10}O_5$ requires C, 57·1; H, 4·8; OMe, 14·8%; equivalent (monobasic), 210].

2:4-Dinitrophenylhydrazone. The mother liquors from the crystallization of the above acid were treated with an excess of Brady's reagent and allowed to stand for 6 hr. The ppt. (m.p. 235–237°) when recrystallized twice from benzene gave orange–red needles of *2-formyl-5-hydroxy-3-methoxy-4-methyl benzoic acid 2:4-dinitrophenylhydrazone*, m.p. 237° (decomp.) [Found: C, 49·5; H, 3·5; N, 14·1. $C_{16}H_{14}O_8N_4$ requires C, 49·2; H, 3·6; N, 14·35%].

Oxidation of quadrilineatin methyl ether with potassium permanganate: isolation of 3:5-dimethoxy-4-methylphthalic acid. The crude methyl ether of quadrilineatin, prepared as described above, in the form of a golden-yellow syrup (0·81 g.), was dissolved in purified acetone (40 ml.), and finely powdered $KMnO_4$ (3·1 g.) was added gradually with shaking until the colour remained for 3 hr. The solution was decolorized with a few drops of ethanol and the MnO_2 removed by filtration. A negligible amount of dissolved material was present in the acetone solution. The MnO_2 was extracted with hot water (3 × 30 ml.) and the aqueous extract was acidified to Congo red with HCl and extracted with ether (4 × ½ vol.). The sticky light-brown residue from the ether was crystallized from ethyl acetate–light petroleum and gave colourless needles (0·20 g.) of m.p. 202–204°, with sublimation and gas evolution, resetting on cooling and remelting at 160–162°. The initial m.p. was raised by recrystallization from water to 207–208°, remelting at 164–165°. The m.p. was not depressed on admixture with authentic 3:5-dimethoxy-4-methylphthalic acid (m.p. 206–207°) synthesized for comparison [Found: C, 54·6; H, 5·2; *C*-Me, 6·7; OMe, 25·6%; equivalent by titration, 120. $C_{11}H_{12}O_6$ requires C, 55·0; H, 5·0. *C*-Me, 6·3, two OMe, 25·8%; equivalent (as dibasic acid), 120].

Conversion into the anhydride. The 3:5-dimethoxy-4-methylphthalic acid (55 mg.) was heated at 220° for 20 min. The product rapidly solidified on cooling. On sublimation in high vacuum at 120–130°, colourless needles (35 mg.) of 3:5-dimethoxy-4-methylphthalic anhydride of m.p. 165–166° were obtained. The product did not depress the m.p. of the authentic anhydride on admixture [Found: C, 58·9; H, 4·5; OMe, 28·0. $C_{11}H_{10}O_5$ requires C, 59·4; H, 4·5; two OMe, 27·9%].

Demethylation and monodecarboxylation of 3:5-dimethoxy-4-methylphthalic acid: isolation of 3:5-dihydroxy-p-toluic acid. The dimethoxy acid (0·10 g.) was refluxed for 20 min. with HBr (46–48%; 5 ml.). All the solid dissolved to form a light-brown solution. Almost colourless needles separated on cooling (50 mg.; m.p. 262–264°). The product was recrystallized from ethyl acetate–light petroleum, yielding microcrystalline needles (30 mg.) of 3:5-dihydroxy-4-methylbenzoic acid, m.p. 264–265° with sublimation and darkening. The m.p. was unchanged after sublimation. The product gave no colour with aqueous or ethanolic $FeCl_3$ and did not depress the m.p. of authentic 3:5-dihydroxy-*p*-toluic acid on admixture [Found (on sublimed specimen): C, 56·15; H, 5·0%; equivalent by titration, 166. $C_9H_8O_4$ requires C, 56·4; H, 4·8%; equivalent (as monobasic acid), 168].

Methyl 3:5-dihydroxy-p-toluate. The above acid (30 mg.) was dissolved in ether and treated with a slight excess of diazomethane. The solvent was removed and the residue was recrystallized twice from benzene to give needles (15 mg.) of methyl 3:5-dihydroxy-*p*-toluate; m.p. 189°, which did not depress the m.p. of an authentic specimen on admixture [Found: C, 59·4; H, 5·8; OMe, 17·2. $C_9H_{10}O_4$ requires C, 59·3; H, 5·5; OMe, 17·0%].

Production of a phthalazine from quadrilineatin

Action of hydrazine hydrate. Quadrilineatin (0·20 g.) and hydrazine hydrate [50% (v/v) prepared from 98–100% hydrazine; 0·10 ml.] were mixed to give a golden-brown solution. Water (3 ml.) was added and the mixture was refluxed for 15 min. The deep-brown solution was concentrated to about half volume on the water bath, when golden-yellow needles separated and were collected (0·15 g., m.p. 225–228°). Recrystallization from aqueous ethanol (50%, v/v) gave slightly yellow needles of *7-hydroxy-5-methoxy-6-methylphthalazine*, m.p. 226–228° (decomp.). These were immediately soluble in cold 2N-HCl and gave no ppt. with Brady's reagent and only a very slight yellowish brown colour with $FeCl_3$ [Found (on a specimen dried 1½ hr. in high vacuum at 100°, no loss in weight): C, 62·1; H, 5·2; N, 14·2; OMe, 16·55. $C_{10}H_{10}O_2N_2,\frac{1}{2}H_2O$ requires C, 61·95; H, 5·4; N, 14·45; OMe, 16·0%].

Action of semicarbazide. Quadrilineatin (0·32 g.) in pyridine (1·6 ml.) was mixed with a saturated solution of semicarbazide hydrochloride in water. Almost at once a crystalline ppt. of short needles, m.p. 195–197° was obtained. Recrystallization from water raised the m.p. to 228°. The product gave no depression when mixed with the phthalazine obtained by means of hydrazine hydrate [Found: C, 62·25; H, 5·6; N, 13·8. $C_{10}H_{10}O_2N_2,\frac{1}{2}H_2O$ requires C, 61·95; H, 5·4; N, 14·45%].

Demethylation of the phthalazine: formation of 5:7-dihydroxy-6-methylphthalazine hydrobromide. This phthalazine (55 mg.) was refluxed with HBr (50%; 3 ml.) for 3 hr. From the golden-brown solution light-brown needles (45 mg.; m.p. 232–234°) separated. Two recrystallizations from ethanol–ether (1:1) gave colourless needles (25 mg.) of *5:7-dihydroxy-6-methylphthalazine hydrobromide*, m.p. 238–240° (decomp.). The product persistently retains ethanol of crystallization [Found (on a specimen dried in low vacuum at room temp.): OEt, 14·4. $C_9H_9O_2N_2Br,C_2H_5{\cdot}OH$ requires OEt, 14·9%. Found (on a specimen dried to constant wt. in high vacuum at 100°): C, 43·1; H, 4·3; N, 9·8; Br, 28·0; OEt, 8·5. $C_9H_9O_2N_2Br,\frac{1}{2}C_2H_5{\cdot}OH$ requires C, 42·9; H, 4·3; N, 10·0; Br, 28·5; OEt, 8·0%]. The m.p. of a specimen prepared by demethylation of 5-hydroxy-7-methoxy-6-methylphthalazine prepared from cyclopaldic acid (see below) was not depressed on admixture with this compound.

Action of dilute alkali on quadrilineatin: production of 6-hydroxy-4-methoxy-5-methylphthalide. Quadrilineatin (0·2 g.) was refluxed for 30 min. with 2N-NaOH (20 ml.). The solution, originally light yellow, became brown in a few minutes. After cooling it was acidified with HCl. The crystalline deposit (0·19 g., m.p. 216–218°) was collected and recrystallized twice from water. *6-Hydroxy-4-methoxy-*

5-methylphthalide was thus obtained as needles (0·15 g.) of m.p. 234–235° (with sublimation). The product gave an immediate stable brown colour with the Gibbs reagent and a pink colour with cold conc. H_2SO_4; on warming with conc. H_2SO_4 it gave a slight purplish tinge, changing to a stable light brown [Found (on a sublimed specimen): C, 61·7; H, 5·2; OMe, 16·0. Calc. for $C_{10}H_{10}O_4$: C, 61·8; H, 5·2; OMe, 16·0%].

Methylation of the phthalide: identification of product as 4:6-dimethoxy-5-methylphthalide. The phthalide (0·1 g.) was dissolved in dry acetone and refluxed for 6 hr. with anhydrous K_2CO_3 (2·5 g.) while methyl iodide (5 ml.) was added in portions. The acetone was removed and the residue suspended in water. Colourless needles (85 mg., m.p. 156–158°) were formed on acidification. These were collected and recrystallized twice from ethanol, giving 4:6-dimethoxy-5-methylphthalide (40 mg.) of m.p. 158°. The m.p. of authentic 4:6-dimethoxy-5-methylphthalide synthesized for comparison, was not depressed on admixture [Found: C, 63·3; H, 5·9; OMe, 30·0. Calc. for $C_{11}H_{12}O_4$: C, 63·4; H, 5·8; (two) OMe, 29·8%].

Identification of high-melting product previously obtained in crystallization of quadrilineatin. It was previously reported by one of us (Lahoz-Oliver, 1955) that small amounts of a second product (product *B*) melting at 225–228° were obtained when toluene was used for recrystallization of crude quadrilineatin. This was thought to be a second metabolic product derived from *A. quadrilineatus*. When the method of crystallization from toluene previously employed was repeated on some of the crude material (1·1 g.), colourless needles (0·6 g.) of quadrilineatin of m.p. 168–170° were obtained as the main crop. The mother liquors on further concentration afforded colourless needles (0·24 g.) of m.p. 228–230°, raised to 235–236° on further crystallization from water with the use of activated carbon. The m.p. was raised to the constant value of 236° by sublimation in high vacuum. This product did not depress the m.p. of the 6-hydroxy-4-methoxy-5-methylphthalide obtained by the action of 2N-NaOH on quadrilineatin [Found (on a sublimed specimen): C, 61·9; H, 5·1; OMe, 15·8: Calc. for $C_{10}H_{10}O_4$: C, 61·8; H, 5·2; OMe, 16·0%]. None of this phthalide was obtained when benzene was used as solvent for crystallization and precautions were taken to avoid overheating. The product must therefore be regarded as an artifact and not as a second metabolite.

Proof of the structure of the Doeuvre-oxidation acid as 6-formyl-4-hydroxy-2-methoxy-3-methyl-benzoic acid

Reduction of the Doeuvre acid: production of a phthalide. The Doeuvre-oxidation acid (0·21 g.) in methanol (5 ml.) was treated with Zn dust (0·2 g.) followed by H_2SO_4 (50%, v/v; 1·5 ml.). The mixture was warmed slightly and shaken intermittently for 15 min., when the solution no longer gave a ppt. with Brady's reagent. The solution was filtered, diluted with water and chilled. It deposited colourless lustrous needles (0·14 g., m.p. 193–195°). Further material (40 mg., m.p. 191–193°) separated as a second crop. The combined material on recrystallization from water formed colourless needles (0·15 g.) of *5-hydroxy-7-methoxy-6-methylphthalide*, m.p. 198° [Found (on material sublimed in high vacuum at 140–150°): C, 61·6; H, 5·1; OMe, 16·2%. Calc. for $C_{10}H_{10}O_4$: C, 61·8; H, 5·2; OMe, 16·0%].

Methylation of the phthalide: formation of 5:7-dimethoxy-6-methylphthalide. The phthalide (50 mg.) was dissolved in dry acetone (15 ml.) and dry K_2CO_3 (2·5 g.) was added. The mixture was refluxed for 6 hr. with addition of methyl iodide (5 ml.) in portions. After removal of the solvent the residue was treated with water and acidified with HCl; crystalline needles separated (45 mg., m.p. 137–138°). The m.p. was raised by recrystallization from water to 139–140°. The methoxyl content at this stage was 32·7%: $C_{11}H_{12}O_4$ (two OMe) requires 29·8%; $C_{12}H_{16}O_5$ (three OMe, assuming ring opening and esterification of the carboxyl group) requires 38·8%. It was probable that some esterification had occurred. The product (25 mg.) was therefore refluxed for 4 hr. with ethanolic KOH (10% solution; 5 ml.). The solution was evaporated with addition of water and acidified. Colourless needles (20 mg., m.p. 169–171°) separated and were recrystallized from water. Sublimation in high vacuum at 100–110° gave 5:7-dimethoxy-6-methylphthalide of m.p. 171·5°. The m.p. of authentic material obtained by a previous synthesis (Birkinshaw *et al.* 1952) was not depressed on admixture with this product [Found: C, 62·9; H, 5·7; OMe, 29·45. $C_{11}H_{12}O_4$ requires C, 63·4; H, 5·8; (two) OMe, 29·8%].

Syntheses

4:6-Dimethoxy-5-methylphthalide. Charlesworth & Robinson (1934) prepared this from 3:5-dimethoxy-*p*-toluic acid in three stages by the method of Fritsch (1897). The following method (chloromethylation) accomplishes the reaction in one step. 3:5-Dimethoxy-*p*-toluic acid (0·2 g.), acetic acid (2 ml.) and chloromethyl ether (0·5 ml.) were heated under reflux at 80° for 8 hr. The initial light-brown solution gradually became pink and crystalline material separated. The mixture was treated with N-HCl (50 ml.) and refluxed for ½ hr. Most of the solid material had then dissolved. The solution was filtered from a little tar and extracted with ether (3 × ½ vol.). The combined ether extracts were shaken with 2N-Na_2CO_3 (2 × 5 ml.). Acidification of the Na_2CO_3 extract afforded unchanged starting material (0·11 g.). From the ether a crystalline residue (0·08 g.) was obtained, which on recrystallization from ethanol afforded needles (45 mg.) of 4:6-dimethoxy-5-methylphthalide, m.p. 158°, the same as that recorded by Charlesworth & Robinson (1934) [Found: OMe, 29·7. Calc. for $C_{11}H_{12}O_4$: (two) OMe, 29·8%].

3:5-Dimethoxy-4-methylphthalic acid. This acid was prepared from the phthalide by $KMnO_4$ oxidation according to the method of Charlesworth & Robinson (1934), who record the m.p. as 195–196° (decomp.). Recrystallization from water gave prismatic rods, m.p. 206–207° with gas evolution and sublimation, resetting and remelting at 165–166° [Found: OMe, 25·75. Calc. for $C_{11}H_{12}O_6$: (two) OMe, 25·8%].

3:5-Dimethoxy-4-methylphthalic anhydride. The acid (25 mg.) was held at 230° for 10 min. and then sublimed in high vacuum at 100–110°. The product was recrystallized from light petroleum, giving colourless needles of the anhydride, m.p. 166°, the same as that recorded by Charlesworth & Robinson (1934) [Found: OMe, 27·65. Calc. for $C_{11}H_{10}O_5$: (two) OMe, 28·0%].

Demethylation of 5-hydroxy-7-methoxy-6-methylphthalazine: formation of the monohydrobromide of 5:7-dihydroxy-6-methylphthalazine. 5-Hydroxy-7-methoxy-6-methylphthalazine (55 mg.), prepared by the action of hydrazine hydrate on cyclopaldic acid as described by Birkinshaw *et al.* (1952),

was refluxed for 2½ hr. with HBr (46–48%; 3 ml.). All the solid had then dissolved. On cooling, light greyish brown needles separated which still contained methoxyl (3·1%). The refluxing with HBr was continued for a further 1½ hr. Needle crystals (35 mg.; m.p. 232–234°) were obtained, of which the methoxyl content was nil. Recrystallization from ethanol–ether (1:1) with charcoal gave colourless needles (22 mg.) of the hydrobromide of 5:7-dihydroxy-6-methylphthalazine, m.p. 238–240° (decomp.), retaining ½ mol. of ethanol even when dried at 100° in high vacuum [Found: C, 43·1; H, 4·2; N, 9·7; Br, 28·2; OEt, 7·9. $C_9H_9O_2N_2Br$, $\frac{1}{2}C_2H_5{\cdot}OH$ requires C, 42·9; H, 4·3; N, 10·0; Br, 28·5; OEt, 8·0%].

SUMMARY

1. *Aspergillus quadrilineatus* Thom & Raper, when grown on Raulin–Thom solution, produces in the culture medium a hitherto undescribed fungal metabolite, which has been named quadrilineatin.

2. Quadrilineatin, $C_{10}H_{10}O_4$, forms colourless needles of m.p. 172° (decomp.). From the study of its functional derivatives and oxidation products, a number of which are described, quadrilineatin has been allocated the structure 1:2-diformyl-5-hydroxy-3-methoxy-4-methylbenzene.

3. Quadrilineatin is thus a member of the group of substituted *o*-phthalaldehydes, of which three, namely gladiolic acid, cyclopaldic acid and flavipin, were previously known as fungal metabolites.

4. Quadrilineatin shows only weak antibacterial activity against *Staphylococcus aureus* and *Escherichia coli*.

The light-absorption values were determined by means of a Hilger Uvispek spectrophotometer purchased by means of a grant from the Central Research Fund of London University.

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APPENDIX

Anti-Fungal Tests on Quadrilineatin

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Quadrilineatin was tested by the method described by Brian & Hemming (1945) and used previously in this Laboratory for determining the fungistatic activity of cyclopaldic acid and related substances. The procedure is to determine the lowest concentration that will inhibit the germination of the conidia of *Botrytis allii* Munn. A series of twofold dilutions in 'germination medium' plus spore suspension were made, starting at 1 in 25 000. There was complete inhibition at the highest concentration only, 1:25 000 (≡40 μg./ml.), partial inhibition up to 1:100 000 and virtually 100% germination at higher dilutions. The substance is therefore considerably less active than cyclopaldic acid, which showed complete inhibition at a dilution of 2·5 μg./ml., and gladiolic acid and flavipin, which inhibited completely at 10 μg./ml.

A second series of tests were carried out to determine the activity of quadrilineatin against the 'damping-off' fungus *Pythium debaryanum* Hesse. A primary series of twofold dilutions in sterile water were made. Tubes containing exactly 14 ml. of Czapek–Dox agar were prepared and sterilized. To each of these, with the agar melted and then cooled to 45°, was added 1 ml. of aqueous solution. The primary dilutions were made so that on dilution 1:15 a series of dilutions in agar starting at 1:12 500 were obtained. The concentration required for the first of the primary dilutions was greater than the solubility of the substance in water. Complete

solution was effected by adding the least possible amount of sodium carbonate. The amount of alkali introduced in this way is too small to affect appreciably the pH of the strongly buffered Czapek–Dox agar.

The melted agar in the tubes was poured into the corresponding number of Petri dishes and allowed to set. Each dish was inoculated centrally with mycelium from a young culture of the *Pythium*. The results were recorded after 3 days' incubation, which is the time taken for a control plate to be completely covered. There was complete inhibition at a dilution of 1:25 000 and partial inhibition at 1:50 000. Thus two fungi belonging to widely different orders are inhibited completely by the same concentration.

SUMMARY

1. The inhibition of fungal growth by quadrilineatin was tested against two species of fungi. Complete inhibition of germination of *Botrytis allii* conidia and of mycelial growth of *Pythium debaryanum* occurred at a dilution of 1:25 000 (40 μg./ml.).

2. Quadrilineatin is a less potent inhibitor of *Botrytis allii* than the other known phthalaldehydes of fungal origin.

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The Flame-Spectrophotometric Determination of Calcium in Biological Fluids and an Isotopic Analysis of the Errors in the Kramer–Tisdall Procedure

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Although chemical methods have been superseded by the flame photometer in the determination of sodium and potassium in biological fluids, calcium is still usually determined chemically. The method in most widespread use is the procedure of Kramer & Tisdall (1921) or one of its modifications (Tisdall, 1923; Clark & Collip, 1925). This method involves the direct precipitation of calcium from serum with ammonium oxalate, isolation by centrifuging, and titration with permanganate after washing with dilute ammonia. This procedure is tedious and has other disadvantages.

Kirk (1954) regards the procedure as one of known unreliability. The same conclusion may be reached from a consideration of the conditions of precipitation which are far from optimum (Kolthoff & Sandell, 1952). Smith *et al.* (1950) showed that magnesium may be co-precipitated to the extent of 10% of the normal calcium content (expressed in equivalents) when serum is analysed. That such accuracy as the method possesses must depend on compensation of errors was well known to Clark & Collip (1925).

That flame photometry has not been widely used for calcium determination is due to two main difficulties. The first is the proximity of the relatively weak calcium oxide bands at 554 and 620mμ to the intense sodium line at 590 mμ. This, together with the preponderance of sodium, makes marked spectral interference inevitable with the relatively simple instruments now in wide use (Powell, 1953). Even when instruments incorporating a monochromator (such as the Beckman flame photometer) are used, this difficulty is not entirely removed (Severinghaus & Ferrebee, 1950). Incorporation of sodium in the standard solutions (Baker, 1955) may allow correction to be made when serum calcium is being estimated, but the variability of sodium concentrations makes this procedure inaccurate when applied to urine.

The second difficulty is the fact that calcium is much more susceptible to interference from anions, in particular phosphate (Brealey, Garratt & Proctor, 1952; Chen & Toribara, 1953; Denson, 1954; Leyton, 1954; Baker & Johnson, 1954), than are sodium and potassium (Shapiro & Hoagland, 1948; Collins & Polkinhorne, 1952; Domingo & Klyne, 1949). As will be shown, neglect of this effect, as by Severinghaus & Ferrebee (1950), may cause serious error. Prior precipitation of calcium as oxalate (Powell, 1953; Llaurado, 1954) should overcome both these difficulties; however, it leads only to combining some of the error of the oxalate procedure with little of the convenience of the flame photometer.

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In view of these difficulties, a suitable flame spectrophometer was constructed (MacIntyre, 1954, 1955) and a study made of those interfering effects of importance in biological fluids. As a result a method was developed for the determination of calcium in plasma and urine, deproteinization being the only prior step necessary.

In view of the claims of Smith *et al.* (1950) and Lehmann (1953) that the traditional figures for normal serum calcium are too high, a series of normal sera were examined, parallel estimations being carried out by the flame photometer and by the Kramer–Tisdall method as described by King (1951), for which an adequate series of normal figures is available (Wootton, King & Smith, 1951). There was only fair agreement between these two methods. In the author's hands the ethylenediaminetetra-acetic acid procedure of Fales (1953), which employs murexide and a spectrophotometric end-point, was too imprecise to cast light on the source of the discrepancy. A similar imprecision was found with a modification of the procedure of Sweetser & Bricker (1954) in which the end point of the ethylenediaminetetra-acetic acid titration is detected by the sudden change in extinction in the ultraviolet.

It was necessary, therefore, to test the flame-photometric method exhaustively, and to attempt to analyse in detail the sources of error in the oxalate procedure. As the use of isotopes provides a reliable tool for investigating analytical errors, ^{45}Ca was employed to measure the losses of calcium in the Kramer–Tisdall procedure during precipitation and washing. Analyses of precipitates for magnesium and phosphorus were also carried out. The results presented are believed to offer further evidence on the unreliability of the oxalate procedure of Kramer & Tisdall.

EXPERIMENTAL

Description of the flame spectrophotometer

The component parts are an atomizer, spray chamber, burner, monochromator and photomultiplier (Fig. 1).

Gas supply. Air and acetylene are supplied from cylinders fitted with two-stage regulators (British Oxygen Co. Ltd., Wembley, Middlesex).

Atomizer. The atomizer is constructed of Perspex, stainless steel and platinum–iridium alloy. It is concentric in type (Fig. 3).

Spray chamber. This consists of a simple glass cylinder with suitable baffles (Fig. 2).

Burner. The burner is that of Baker (1955). It is held in an adjustable holder allowing both vertical and lateral adjustment. Precise vertical adjustment is important to ensure maximum sensitivity.

Monochromator. This is the Hilger Barfit Wavelength Spectrometer D. 186, which uses a constant-deviation glass prism (Hilger and Watts Ltd., London, N.W. 1).

Photocell. This an RCA-931 A multiplier phototube (RCA Photophone Ltd., Sunbury-on-Thames, Middlesex). It is placed in the compartment in place of the phototube

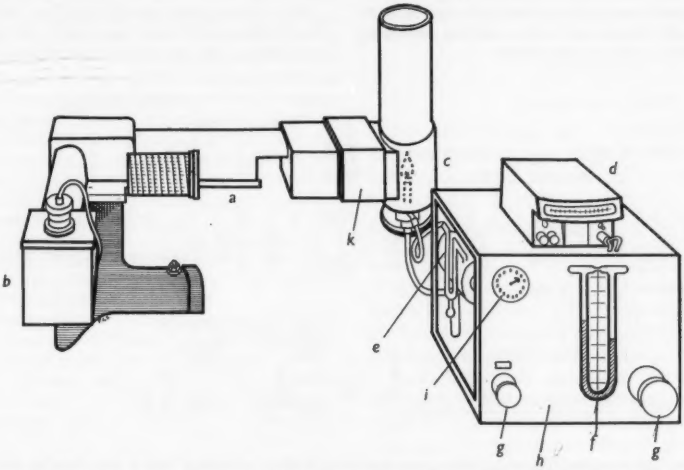

Fig. 1. Flame spectrophotometer (semi-diagrammatic). The main components (monochromator *a*, burner and chimney *c*, and metal box *h*) are screwed to a wooden base not shown in the figure. The metal box houses the spray chamber *e*, flowmeter *f*, rubber-tubing connexions and air-pressure gauge *i*. The galvanometer *d* rests on top of *h*, needle valves *g*, controlling air and acetylene, are set in the front panel. There is a light-tight metal connexion *k* between *c* and *a* which serves to exclude extraneous light from the entrance slit. The phototube housing *b*, contains the photomultiplier.

supplied with the spectrometer. During the course of this work it was operated at 1080 v supplied from batteries. The photomultiplier cathode is connected to the battery cathode. The last dynode is connected to the battery anode and to earth. 120 000 Ω resistors are connected between each dynode. The galvanometer measures the current flowing between the last dynode and the photomultiplier anode. Since the work was completed the phototube has been operated at 1200 v, with a consequent increase in sensitivity.

Galvanometer. The instrument used for this work was a Scalamp galvanometer (sensitivity 90 mm./μA) (W. G. Pye and Co. Ltd., Cambridge). This has now been replaced with a Scalamp galvanometer of double this sensitivity.

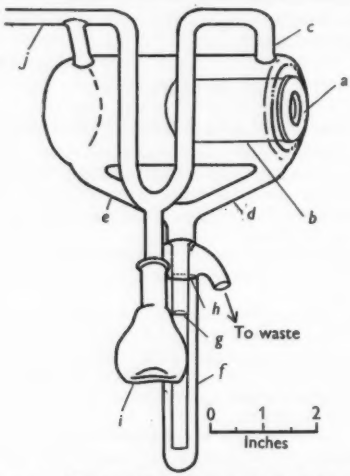

Fig. 2. Spray chamber. This is constructed of glass except for the brass plate *a*, into which is screwed the atomizer. A cylinder *b* prevents large droplets reaching *c*, the exit tube from the chamber. Two limbs *d* and *e* conduct condensed liquid to the constant-pressure drain *f*. The two fluid levels *g* and *h* are shown. Overflow from *h* is led to waste. The small detachable bottle *i* collects condensed fluid from *c*. Tube *j* leads to the burner.

Operation of the flame spectrophotometer

The air pressure is adjusted to 16 lb./sq.in. and the aspiration rate adjusted to 4–6 ml./min. The acetylene pressure is adjusted to 4 lb./sq.in. at the cylinder and the flowmeter adjusted so that the rate is about 500 ml./min.

The flame is now lighted and the monochromator entrance and exit slits are adjusted. Slit widths of 0·2 mm. were used for calcium estimations. The greater sensitivity since obtained now allows the use of 0·06 mm. slit widths.

The wavelength control is set at the desired figure (423 mμ for Ca). The photomultiplier circuit is completed and the galvanometer switched to full sensitivity. A suitable solution, e.g. the 0·25 mM-calcium standard, is sprayed and the wavelength setting checked to ensure that maximum sensitivity is achieved. The acetylene adjustment is slowly altered to a setting which gives maximum sensitivity. This setting is also the one least affected by any possible variations in the acetylene flow rate.

Distilled water is sprayed for about 10 min. At the end of this period steady reproducible deflexions are constantly obtained and the instrument is ready for use. Distilled water is repeatedly sprayed between the sprayings of test and standard solutions.

Determination of interference effects

The solutions used were all made from AnalaR chemicals. The distilled water used was passed through a column of mixed anion- and cation-exchange resins (Amberlite MB-1).

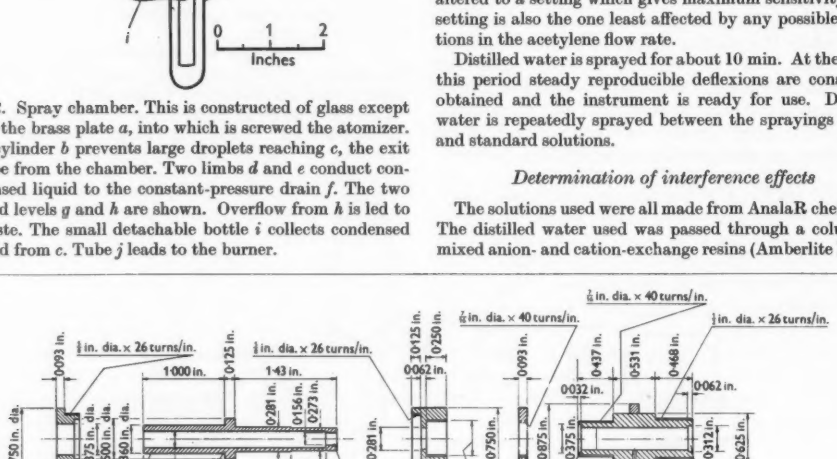

Fig. 3. Atomizer. The sectional drawing shows the retaining ring *a*, adjusting ring *b* and locking ring *c*. These components are made of brass. The stainless-steel tube *d* fits tightly inside the Perspex inner tube *e*. The platinum–20% iridium tip *f* fits into *g*. The platinum–20% iridium disk *i* fits into the body of the atomizer *h* at *j*. A rubber O-ring seal fits into *m*. The test solution enters the body of the atomizer via the polythene tube *n* and Perspex inlet-tube *k*. The Perspex inner tube fits inside the body. A rubber connexion from the air cylinder fits over *e* at *l*. The rate of aspiration is determined by the distance between *f* and *i*. This is controlled by *b*, which, together with *a*, advances *e* into *h*. A satisfactory position is maintained by locking ring *c*.

The standard calcium solution was made from AnalaR $CaCO_3$ (British Drug Houses Ltd., Poole, Dorset), dried at 350° and dissolved in the minimum amount of hydrochloric acid. As with the standard solutions used for blood and urine analyses, polythene containers were used for all solutions kept more than a few hours.

All glassware used was washed in dichromate–sulphuric acid cleaning mixture, and then rinsed thoroughly in several changes of distilled water.

All solutions were kept from contact with rubber bungs or filter paper.

The procedure for determining interference effects was to record the galvanometer deflexions produced by various solutions of the same calcium content, containing increasing amounts of the constituent being examined. 'Blank' readings without calcium were recorded in every case, and in addition a standard solution was sprayed between each pair of test solutions. This enabled any slight change in the sensitivity of the instrument to be detected. Where this occurred the readings were corrected.

Determination of calcium in biological fluids

All solutions were made with deionized distilled water.

Stock solutions. These were as follows. Calcium solution: 25 mM-$CaCl_2$; mixed salt solution: 30 mM-KCl, 5 mM-K_2SO_4, 1·4 M-NaCl, 50 mM-KH_2PO_4; magnesium solution: 8 mM-$MgCl_2$; perchloric acid: $HClO_4$ 60% (w/w); phosphate solution: 44·4 mM-KH_2PO_4. Working standards were prepared by adding 10 ml. of mixed salt solution, 10 ml. of magnesium solution and an appropriate volume of calcium solution to 700 ml. of water in a litre flask. Perchloric acid (50 ml.) was now added, and after mixing the volume was made to a litre. A range of standards at intervals of 0·025 mM was prepared from 0·05 mM to 0·5 mM with respect to calcium concentration. The constituents of the working standards were chosen to approximate to the mean plasma filtrate concentrations after deproteinization of 1 vol. of plasma with 9 vol. of a phosphate–perchloric acid solution. This deproteinizing solution contained sufficient phosphate to eliminate the interfering effect of varying plasma concentrations. The 0·05 mM-sulphate in the working standard solutions is somewhat higher than that suggested by the normal figures of Letonoff & Reinhold (1936), namely 0·03 mM. However, these authors consider that acid deproteinizing reagents liberate an increased amount of inorganic sulphate from protein.

In general, the amounts of the various salts added were adjusted to minimize the interference effects. They were such that the variation of these constituents which occurs in biological fluids would not affect the results.

Combined diluting and deproteinizing reagent. Phosphate solution (10 ml.) was diluted to 700 ml., mixed with 55·5 ml. of perchloric acid and made up to 1 l. with water.

Plasma or serum was prepared for analysis by adding 1 vol. to 9 vol. of deproteinizing reagent and centrifuging, the clear supernatant being used. Urine (1 vol.) was added to 18 vol. of deproteinizing reagent and 1 vol. of water. The diluted urine was centrifuged if necessary before analysis. It is advisable to collect 24 hr. samples of urine in Winchester containers, to which 10 ml. of concentrated HCl has been added. This prevents precipitation of calcium as phosphate before analysis.

It was found that the galvanometer deflexion was related linearly to calcium concentration. However, a small deflexion is produced at 423 mμ by the other constituents of the standard, owing to a small extent to the NaCl (spectral interference is of the order of 2% for a normal serum calcium) and to a larger extent to the perchloric acid, which gives a low-intensity continuous spectrum. Therefore at least two standards were sprayed for each test solution, and the final answer was arrived at by interpolation. Two readings of test and each of two standards were taken.

The direct oxalate precipitation procedure of Kramer & Tisdall (1921) as described by King (1951) was compared with the flame-spectrophotometric method in a series of twenty-two normal and nine pathological sera. Half an hour was allowed for precipitation.

Investigation of the Kramer–Tisdall procedure

The losses of Ca in precipitation and washing were investigated with ^{45}Ca. Calcium acetate (1 μc) of specific activity of 1 c/g. was added to each of ten 2 ml. serum samples. The sera were then set aside for 12 hr. The calcium was precipitated and washed by the same technique as in the comparison series. Samples of the dissolved precipitate, 'decalcified' serum, and washings were now counted after drying on planchets. The addition of the calcium acetate involved only a negligible change in the total calcium content of the sera (about 0·5%).

In eight of these sera, samples of the calcium oxalate precipitate were analysed for Mg content after dissolution in nitric acid and evaporation to dryness. The method of Orange & Rhein (1951) was used. In the author's hands this method had a precision of 5%. A standard was prepared containing an amount of calcium equivalent to that in the test sample. The mean phosphate content of these precipitates was found by pooling and analysing by the method described by King (1951).

In addition oxalate precipitates were obtained from a further eight sera and analysed individually for phosphate content.

RESULTS

Interference effects

Inorganic constituents. Interference effects were studied in the greatest detail with 0·25 mM-$CaCl_2$ but other concentrations within the range 0·05–0·50 mM showed corresponding effects. The emission spectrum of a solution containing 14 mM-NaCl and 0·25 mM-$CaCl_2$ was studied; these are the proportions which exist in human plasma. The proportions of the total emission due to Ca, Na and the flame were: 71·5, 1·5 and 27% respectively at 423 mμ; 80, 8 and 12% respectively at 554 mμ; and 82, 12·5 and 5·4% respectively at 624 mμ.

Both Na and K caused an increase in emission of Ca. In neither case was spectral interference responsible for more than a minor part of this effect. This increase remained constant at 5% for concentrations of Na and K from 3·0 to 20·0 mM. Magnesium chloride was without effect in the concentrations examined from 0·05 to 0·20 mM.

In Fig. 4 the effect of phosphate is shown. There is a marked depression of calcium emission, reaching a maximum when the P:Ca atomic ratio is about

1:1·25. Further increase in the phosphate concentration is without effect.

With 0·25 mM-$CaCl_2$, containing also 14 mM-NaCl, 0·4 mM-KCl and 0·4 mM-KH_2PO_4, K_2SO_4 produced no detectable depression of calcium emission between concentrations of 0·02 and 1·0 mM. Although sulphate is usually reported to depress flame emission, Baker & Johnson (1954) showed that in the presence of excess of phosphate the depressing effect of sulphate was not additive.

The effect of $HClO_4$ and HCl are shown in Fig. 5.

Organic constituents. Neither 0·75% bovine albumin nor 0·75% glycine produced any effect on the emission of 0·25 mM-$CaCl_2$. However, in the presence of 0·4 mM-KH_2PO_4 the albumin, but not the glycine, produced a greater calcium emission than was found with 0·25 mM-$CaCl_2$ and 0·4 mM-KH_2PO_4 alone. A similar effect was obtained by Chen & Toribara (1953).

The adequacy of the plasma-deproteinization procedure in recovering the calcium is shown in Table 1. It is evident from columns (*c*) and (*d*) that the combined effect of non-protein organic constituents and the slight concentration of calcium due to the volume displacement of plasma proteins is not appreciably more than 1%.

The interference of organic substances in urine is shown to be small in Table 2.

Recovery experiments

The adequacy of recovery of calcium added to serum and urine is shown in Tables 3, 4*a* and 4*b*. [The Kramer–Tisdall procedure gave unsatisfactory recoveries when applied to urine (Table 4*a*).]

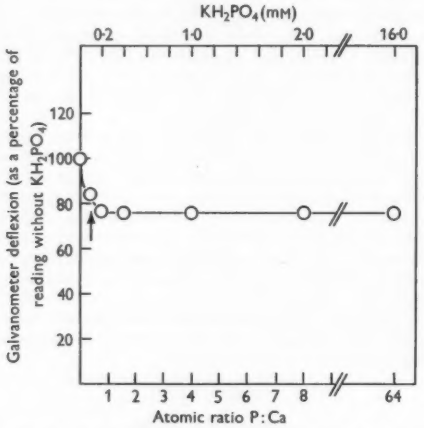

Fig. 4. Effect of phosphate on the emission of calcium. λ 423 mμ; 0·25 mM-$CaCl_2$ used. 16·0 mM-KH_2PO_4 alone produced an emission of 5% of that due to 0·25 mM-$CaCl_2$. The lesser concentrations of KH_2PO_4 tested produced no detectable emission. The arrow indicates mean normal serum concentration.

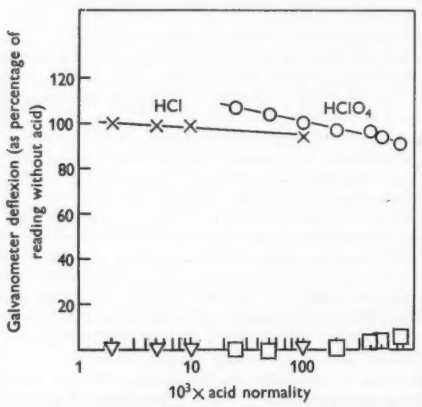

Fig. 5. Effect of hydrochloric and perchloric acids on calcium emission. λ 423 mμ; 0·25 mM-$CaCl_2$ containing also 14·0 mM-NaCl, 0·40 mM-KCl and 0·40 mM-KH_2PO_4 was used. ×, $CaCl_2$ + HCl; ▽, HCl alone; ○, $CaCl_2$ + $HClO_4$; □, $HClO_4$ alone.

Table 1. *Effect of ashing, direct dilution with water and deproteinization on the apparent calcium content of serum*

(*a*) 1 ml. of serum was ashed, dissolved in 2 ml. of 0·1 N-HCl, 1 ml. of 4 mM-KH_2PO_4 and 7 ml. of water.
(*b*) 1 ml. of serum was added to 2 ml. of 0·1 N-HCl, 1 ml. of 4 mM-KH_2PO_4 and 6 ml. of water.
(*c*) 5 ml. of diluted ashed sample as in (*a*) to which 0·3 ml. of 60% (w/w) $HClO_4$ was added.
(*d*) 5 ml. of serum diluted as in (*b*), to which was added 0·3 ml. of 60% $HClO_4$ before centrifuging to remove the protein.

Sample no.	Galvanometer deflexion (mm.) Ashed (*a*)	Direct dilution (*b*)	Ashed + $HClO_4$ (*c*)	Deproteinization with $HClO_4$ (*d*)
1	46·5	54·0	43·0	43·0
2	44·5	53·0	41·0	41·5
3	44·5	52·5	41·5	42·0
4	47·5	54·5	44·0	44·5

Table 2. *Effect of ashing on the apparent calcium content of urine*

The samples were ashed in platinum crucibles at a temperature of 420° before analysis by flame spectrophotometry.

Description of urine sample	Ashed (m-equiv./l.)	Direct estimation (m-equiv./l.)
Normal	12·90	12·95
Normal	4·00	4·00
Proteinuria (0·5 g./100 ml.)	2·55	2·57
Bile + urobilin	2·55	2·40
Glycosuria (6 g./100 ml.)	21·40	22·80

Table 3. *Recovery of calcium added to serum*

Varying quantities of calcium were added to serum no. 1. The analyses were carried out in duplicate by the flame spectrophotometer. The serum used was a pooled specimen of five normal sera.

Sample no.	Obtained (m-equiv./l.) *a*	Expected (m-equiv./l.) *b*	*a* − *b* (m-equiv./l.)	(100*a*/*b*)
1	4·45	—	—	—
2	5·25	5·34	−0·09	98·3
3	5·85	5·78	+0·07	101·2
4	6·25	6·21	+0·04	100·7
5	6·42	6·36	+0·06	101·0
6	6·91	7·03	−0·12	98·3
7	7·36	7·43	−0·07	99·0
Mean (±S.E.M.)			−0·018±0·034	99·8±0·55

Table 4*a*. *Recovery of calcium added to urine*

The analyses were carried out by the flame-spectrophotometer (f.s.) and by the Kramer–Tisdall (K.T.) procedures.

Sample no.	*a* Obtained by K.T. (m-equiv./l.)	*b* Obtained by f.s. (m-equiv./l.)	*c* Expected (m-equiv./l.)	100*a*/*c* (K.T.)	100*b*/*c* (f.s.)
1	1·18	1·18	—	—	—
2	3·15	3·40	3·50	90·0	97·2
3	4·10	4·42	4·43	92·5	99·8
4	5·00	5·57	5·60	89·3	99·5
5	6·05	6·95	6·55	92·4	106·2
6	7·20	8·13	8·13	88·6	100·0
7	8·95	9·47	9·30	96·2	101·9
8	12·10	12·13	12·45	97·2	97·4
Mean (±S.E.M.)				92·3 (±1·35)	100·3 (±1·05)

Table 4*b*. *Recovery of calcium added to urine*

Calcium solution to increase the concentration by 2·50 m-equiv./l. was added to 12 urines. The samples were analysed by the flame spectrophotometer.

	$\frac{\text{Result obtained}}{\text{Result expected}} \times 100$			
No. of expts.	Mean	Range	S.D.	S.E.M.
12	100·6	95·0–103·0	2·07	0·62

The figure in the second column does not differ significantly from 100·0 (*t* test).

The precision of the flame-spectrophotometric method is 0·06 m-equiv. of Ca/l. This was calculated from duplicates of the seven serum analyses shown in Table 3, and eight urine analyses in Table 4*a* from the formula $\sqrt{(\Sigma d^2/2n)}$, where d is the difference between duplicates and n is the number of pairs.

Comparison with the Kramer–Tisdall method

Table 5 shows the comparison with the Kramer–Tisdall procedure.

In the twenty-two analyses on normal sera, the flame-spectrophotometric procedure gave a mean deviation of $+0{\cdot}19 \pm 0{\cdot}049$ m-equiv./l. from the oxalate procedure. When the remaining analyses from the 'pathological' sera are included, the mean deviation is $+0{\cdot}28 \pm 0{\cdot}052$ m-equiv./l. Both these deviations are highly significant ($P < 0{\cdot}001$, t test).

Investigation of the Kramer–Tisdall method

In Table 6 are presented the results of studies on the composition of the precipitates from ten sera, by using the isotopic method of analysis described above (p. 167). This shows that 0·8–2·0 % of the Ca originally present was not precipitated, and a further 2·0–8·9 % was lost in washing. The total loss in this series varied between 2·8 and 10·9 %. The results of analyses for magnesium on eight of the ten precipitates are also presented. The extent of magnesium precipitation was extremely variable and sometimes considerable, and the mean coprecipitation of phosphorus was almost negligible. Coprecipitation of phosphate may on occasion be of more significance, since in another series of eight precipitates the mean phosphorus content was 1·1 μg. (range 0·4–3·5 μg.). This would result in a mean negative error of 0·65 % (range 0·2–2·1 %).

When the negative errors due to Ca^{2+} loss are contrasted with the positive errors due to coprecipitation of Mg, the Ca loss outweighs the Mg loss by 3·3 % (range −7·9 to +7·2 %). The exact magnitude of this figure may not be representative, in a small series, since the coprecipitation of Mg was so variable, but this result is in good agreement with the series of comparisons between the flame-photometric and Kramer–Tisdall methods shown in Table 5.

DISCUSSION

Interference effects

The calcium line in the blue is much more suitable for use in the flame photometry of biological fluids than the bands in the green and red spectral regions, if a suitable photocell is available. When combined with a monochromator, use of this line effectively eliminates the spectral interference of sodium.

Table 5. *Comparison of the flame-spectrophotometric and Kramer–Tisdall procedures*

Thirty-one sera were analysed—twenty-two from normal subjects and nine from those with diseases affecting calcium metabolism.

	No. analysed	Flame spectro-photometer (m-equiv./l.)	Kramer–Tisdall (m-equiv./l.)	Mean diff.	S.E.M. diff.	P (t test)
Normal sera	22	5·39	5·20	0·19	0·049	<0·001
Including abnormal sera	31	5·48	5·20	0·28	0·052	<0·001

Table 6. *Analysis of errors in the Kramer–Tisdall procedure*

The precipitates from ten sera were analysed. The figures for percentage coprecipitation of Mg and P were calculated in equivalents, assuming the calcium to be the mean normal (5·39 m-equiv./l.). The Mg was assumed to be precipitated as oxalate and the phosphate as $CaHPO_4$. The net error was calculated as total Ca loss minus Mg coprecipitation.

Sample no.	Ca loss: Precipitation (%)	Ca loss: Washing (%)	Ca loss: Total (%)	Mg coprecipitation (%)	Net error (%)
1	1·6	6·2	7·8	—	—
2	1·2	6·7	7·9	0·0	−7·9
3	1·5	4·7	6·2	0·7	−5·5
4	1·3	4·9	6·2	0·0	−6·2
5	2·0	8·9	10·9	11·0	+0·1
6	1·3	5·3	6·6	0·0	−6·6
7	0·8	2·0	2·8	10·0	+7·2
8	1·5	6·9	8·4	—	—
9	1·0	3·9	4·9	2·3	−2·6
10	1·2	5·1	6·3	2·3	−4·0
Mean	1·3	5·5	6·8	3·3	3·2

The mean phosphate content of precipitates 2–10 (pooled) was such as to cause a negative error of 0·22 %.

The enhancement effects due to sodium and potassium are small and constant over the biological ranges.

The most marked interference found was that due to phosphate. That it becomes maximal at a P:Ca atomic ratio of about 1:1·25, remaining constant at higher ratios, is fortunate, since it allows elimination of phosphate interference by diluting all samples with phosphate solution. Brealey *et al.* (1952), Leyton (1954), Denson (1954) and Baker & Johnson (1954) have reported a similar effect. The amount of phosphate in plasma is only about half of the minimum amount required to ensure calcium results that are independent of further phosphate increase. This means that incorporation of the mean plasma–phosphate content in the standard solution is not an effective method of eliminating error from phosphate since the emission of calcium in deproteinized plasma will vary inversely with the plasma phosphate. Failure to add any phosphate to standard solutions, however, especially when deproteinized plasma is analysed, inevitably gives results which are too low. Severinghaus & Ferrebee (1950) used such a procedure, and reported excellent recoveries, as might be expected from the results of Leyton (1954). His systematic studies of phosphate interference show that good recoveries would be expected in the analysis of deproteinized plasma by comparison with phosphate-free standards; although the original calcium content would be underestimated.

The use of the internal-standard procedure with lithium (Baker, 1955) is almost certainly an unsound method of dealing with phosphate interference. Even if the lithium emission is subject to an identical degree of depression by phosphate, it is improbable that lithium will be bound by protein to exactly the same extent as calcium. The presence of protein would therefore affect the lithium and calcium emissions to a different degree.

Magnesium and sulphate were without detectable influence on calcium emission in the concentrations tested, but were nevertheless incorporated in the standard solutions. This was done to compensate for any combined interference effect of the plasma salts which had not been detected when their effects were studied individually.

Comparison with the Kramer–Tisdall method

The mean difference of 0·28 ± 0·052 m-equiv./l. in the serum-comparison series cannot be attributed to the effect of the concentration of calcium consequent upon deproteinization. From consideration of the specific volume of plasma proteins (Van Slyke *et al.* 1950), together with the amount of non-solvent water associated with similar proteins (Höber, 1945), it would appear that the volume displacement of proteins should not cause a positive error greater than 0·6%. However, even if the calcium values obtained by flame photometry are reduced by 1% the differences remain highly significant.

The Kramer–Tisdall procedure

The original Kramer–Tisdall method published in 1921 advocated three washings with dilute ammonia, each wash being removed by aspiration. The modification of Tisdall (1923), which is substantially the method used in the comparison series, substituted decantation for aspiration, and two washings with ammonia were considered sufficient. In the modification of Clark & Collip (1925), one washing with ammonia was employed after 5 min. drainage of the centrifuge tube in an inverted position. Clark & Collip were not able to recover calcium added to serum by using the modification of Tisdall (1923). Their own method gave 100% recovery, but it did not agree with the Tisdall modification.

The absence of any generally accepted conclusion about which, if any, of these methods is accurate may have been due to the lack of a sound method of detecting and measuring analytical errors. The use of radioactive tracers does provide such a method—one which is certain to be increasingly used in biochemical analysis (e.g. Bothwell & Mallett, 1955). The results (obtained with ^{45}Ca) presented here show that such errors in coprecipitation of magnesium as may occur, even assuming all the magnesium to be present as oxalate, are less than the errors due to loss of calcium in precipitation and washing. Further, as pointed out by Kolthoff & Sandell (1952), addition of ammonium oxalate to a neutral calcium solution produces a precipitate of uncertain composition, contaminated with basic calcium oxalate, or calcium hydroxide, and tends to give low results. These authors also point out that in the presence of ammonia and phosphate some of the magnesium may be precipitated as magnesium ammonium phosphate. The errors due to precipitation of calcium as phosphate or to inclusion of non-oxalate reducing substances in the precipitate seem to be of the same magnitude (0·5–1·0%) as judged from the analyses of phosphate reported here and from Sendroy's (1944) results. Since the errors tend to cancel each other out, they are not considered in the discussion above.

Rothlin & Bidder (1945) were unable to obtain complete precipitation of calcium directly from serum, and consistently found large mechanical losses in decantation and washing: their results are in good agreement with those reported here.

The isotopic studies afford direct proof of the magnitude of the errors and justify the conclusion that the major part of the discrepancy between the flame-photometric and chemical results should be

attributed to error in the chemical method. The evidence further suggests that the normal values for serum calcium are some 3–4 % higher than the figures given by King (1951).

SUMMARY

1. A flame-spectrophotometric method of estimating calcium in biological fluids is described.

2. Spectral interference due to sodium is mostly eliminated by measuring the calcium emission at 423 mμ.

3. Phosphate interference is overcome by diluting the samples with excess of phosphate.

4. Studies with radioactive calcium (^{45}Ca) show that about 7 % of calcium in serum is lost during washing and precipitation in the procedure of Kramer & Tisdall (Tisdall, 1923).

5. Magnesium is coprecipitated in this procedure in a variable amount (range 0–11 %), averaging 3 %.

6. It is concluded that the accepted normal serum-calcium figures are approximately 4 % too low.

The author wishes to express his thanks to Professor King for suggesting this investigation, and for subsequent advice and criticism.

The flame spectrophotometer was constructed for me by Mr S. Roberts.

Mr A. Asatoor and Miss Doreen Ailward gave much technical assistance.

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Acidic Peptides of the Lens

2. THE USE OF ION-EXCHANGE RESINS AS MOLECULAR SIEVES*

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A tripeptide, named ophthalmic acid, has recently been isolated from calf lens (Waley, 1956). Further work on the structure of ophthalmic acid needed more material than was available from the isolation already carried out. The original method used dialysis, followed by zone electrophoresis, to obtain acidic compounds of low molecular weight; large volumes of solution have to be concentrated to isolate the diffusible compounds, and zone electrophoresis is limited to handling fairly small amounts of material. The procedure described below avoids these drawbacks, has permitted the isolation of ophthalmic acid in the crystalline state, and has allowed the identification of another peptide, norophthalmic acid. This procedure utilizes ion-exchange resins to effect separations based on molecular size as well as ionic charge.

* The paper by Waley (1956) is to be regarded as Part 1.

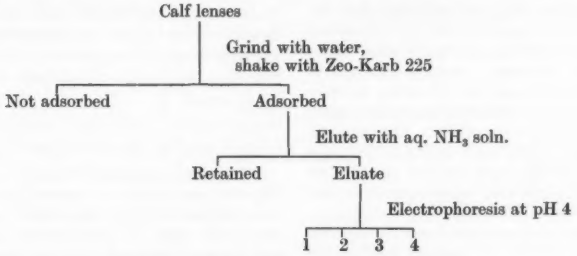

Fig. 1. Scheme of fractionation in Expt. I.

Richardson (1949, 1951) and Kunin (1949) have shown that large ions may not be adsorbed on ion-exchange resins, and Partridge (1952) has pointed out the usefulness of this 'molecular sieve' effect for the separation of small and large ions; amino acids were separated from a protein by using a cation exchange resin (Thompson, 1952). Similarly, Deuel, Solms & Anyas-Weisz (1950) used an anion-exchange resin to separate galacturonic acid from polygalacturonic acid.

METHODS AND RESULTS

General

The methods used were generally the same as those previously described (Waley, 1956). The chromatographic solvent mixture butanol–acetic acid–water (40:9:20, by vol.) is referred to as butanol–acetic acid, and 72% (w/w) phenol–3% (w/v) NH_3 soln. as phenol. Numerical R_F values are not recorded, as the solvent was allowed to drip off the edge during an overnight run on Whatman no. 4 paper; in both these solvents ophthalmic acid had the same R_F as alanine, and norophthalmic acid the same as glycine.

Amino acid estimations on paper were carried out after chromatography on Whatman no. 52 paper; the ninhydrin reagent was that described previously. The same volume (5 μl.) of both the solution and standard solutions was run on the same sheet. Finally, the sheet was cut into strips, parallel to the direction of the solvent flow, and the maximum intensity of the spots measured at 510 mμ, with the apparatus of Tennant, Whitla & Florey (1951) (obtained from K. Kniazuk, 614 Franklin Street, Elizabeth, New Jersey, U.S.A.) in a Beckman Spectrophotometer, Model DU.

Experiment I

The scheme of fractionation in this experiment is set out in Fig. 1.

Adsorption on cation-exchange resin. The resin used was that recommended by Partridge (1952): Zeo-Karb 225 (Permutit Co., London), 15–30 mesh, water-regain 0·75 g./g. Calf lenses (11 g.; stored in a frozen condition), and a little sand, were ground with water (30 ml.), the resin (4·2 g.) was added and the mixture stirred for 1 hr. The supernatant was decanted and the resin washed with water, transferred to a burette and eluted with aq. 3% (w/v) NH_3 soln. until the ninhydrin reaction (tested on filter paper, after removal of the NH_3 in a current of warm air) was no longer positive. The eluate was evaporated to give a yellow syrup (38 mg.).

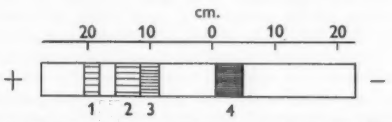

Fig. 2. Electrophoretic fractionation of aq. NH_3 eluate, Expt. I. No. 3 paper; pH 4; 29 v/cm.; 1·7 hr. The depth of shading represents the strength of the ninhydrin colour reaction.

Electrophoretic fractionation. The syrup (38 mg.) in water (0·2 ml.) was applied as a narrow band (14 cm. long) along the starting line (equidistant from the ends and parallel to the shorter side) of a sheet (59 cm. × 20 cm.) of Whatman no. 3 paper. Electrophoresis was carried out at pH 4, with the polythene-covered marble slab previously described; the initial voltage was 2200, but had to be lowered during the run to prevent overheating; after 100 min. the run was stopped (at this stage the voltage was 1100, and the current 115 mA) and a print taken (Fig. 2). The three acidic bands were eluted with water and the eluates evaporated; the residues weighed 1·7 mg. (fraction 1), 1·3 mg. (fraction 2), 4·5 mg. (partly crystalline, fraction 3).

In Fig. 2 band 4 contains neutral species, and the basic compounds have migrated off the paper. Fraction 1 is glutathione (in the disulphide form; oxidation occurs in the alkaline solution used to elute the Zeo-Karb 225 column), fraction 2 consists mainly of ophthalmic acid, and fraction 3 is glutamic acid. These assignations, based on experiments in which these compounds were run alone and in mixtures, were confirmed by paper chromatography in phenol; in this solvent the ratios of the R_F values to the R_F of alanine are, approximately: glutathione, 0·25; glutamic acid, 0·5; ophthalmic acid, 1. As calf lens is reported to contain about 200 mg. of glutathione/100 g. of lens (Kleifeld & Hockwin, 1956), the losses of glutathione in this method are high.

Experiment II

Preliminary experiments were carried out to measure the recovery of ophthalmic acid after adsorption by an anion-exchange resin. The resin [Amberlite IR-4B(OH), analytical

grade, British Drug Houses Ltd.] was converted into the acetate form by stirring with an excess of 5% (v/v) acetic acid for 1 hr., and then washing thoroughly with water. Ophthalmic acid (1 mg.) in water (5 ml.) was added to Amberlite IR-4B acetate (0·5 g., wet wt.), the mixture shaken for 3 min., and the supernatant then shaken again successively with two further batches of resin. The resin was washed with water and then each batch shaken in turn with N-HCl (3 ml.), 2N-HCl (4·5 ml.) and 3N-HCl (5 ml.). The opthalmic acid recovered in the acid extracts, measured by the ninhydrin reaction (Troll & Cannan, 1953), was 0·7 mg., and more might have been recovered on further extraction with acid. This was essentially the method used in the fractionation shown in Fig. 3.

Adsorption on anion-exchange resin (IR-4B). Calf lenses (100 g.) were macerated with water (250 ml.) for 2 min. Amberlite IR-4B acetate (40 g.) was added, the mixture stirred for 10 min., the supernatant decanted on a second lot of resin and stirred, and the supernatant again stirred with a third lot of resin and then rejected. The first lot of resin was stirred with water (250 ml.), the supernatant decanted on the second lot and stirred, and the supernatant again stirred with the third lot of resin before being rejected. This washing was repeated twice more. The third wash was no longer turbid. The third lot of resin was transferred to a tube (diam. 4 cm.), followed by the second lot, and then the first lot, which was thus at the top of the column. The column was eluted with 2N-HCl at 250 ml./hr. for 3 hr., by which time the ninhydrin reaction had become faint. Portions (250 ml.) of the eluate were shaken with *N*-methyldioctylamine in $CHCl_3$ (5% w/v; 2·5 l.) to remove HCl (Smith & Page, 1948; Hughes & Williamson, 1951), and then with $CHCl_3$ to remove excess of base. The eluate had pH of about 5 and was conveniently stored at this stage in the refrigerator (bacterial growth was prevented by the solution's being saturated with $CHCl_3$).

When the solutions were examined by paper electrophoresis at pH 4 and paper chromatography in phenol, the main ninhydrin-positive components seemed to be glutamic acid, glutathione and ophthalmic acid. Other acidic species would, however, be expected to be present. The next step in the fractionation was designed to separate amino compounds by adsorption on a cation-exchange resin; several phosphorus-containing compounds (which are not adsorbed) were thus removed.

Adsorption on cation-exchange resin. The resin (Dowex 50, 200–400 mesh, 12% cross-linked, Microchemical Specialties Inc., California) (70 g.) was washed with 4N-HCl (600 ml.), then with water (3 l.) and made into a column 6·5 cm. long × 4 cm. The eluate (750 ml.) from the anion-exchange column (freed from HCl as described above) was percolated through the column at 80 ml./hr., and the column then washed with water (750 ml.). Elution was effected with 3% (w/v) NH_3 solution, the eluate being collected in 35 ml. fractions; the rate of elution was 180 ml./hr. The ninhydrin-positive material (tested on paper, as described in Expt. I) was found in fractions 5–8, which were combined and concentrated to a volume of about 3 ml., and filtered to remove a brown solid.

The chief advantage gained by this stage of the fractionation was that the paper chromatography gave a much cleaner chromatogram than previously, presumably owing to the removal of other acidic species. There were no spots in positions corresponding to glutamic acid, glutathione or ophthalmic acid in the aqueous solution flowing through the cation-exchange column; so these compounds had, as expected, been quantitatively retained by the column.

Chromatographic fractionation. Whatman no. 3 paper was washed chromatographically with acid and alkali as described by Fowden (1956); a final wash with ethanol was also carried out to hasten drying.

The solution described above (3 ml.) was spread along the full width of two washed no. 3 papers (22½ in. × 18¼ in.);

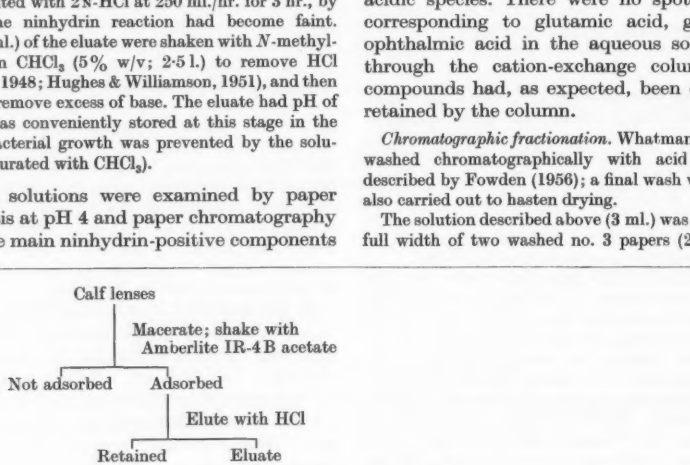

Fig. 3. Scheme of fractionation in Expt. II.

repeated applications (drying in between) ensured a narrow band. The chromatograms were run with phenol as solvent for 17 hr., and prints then taken; narrow strips (their long directions parallel with the direction of flow) were also cut out of the middle of both sheets, and these strips and the prints were developed with ninhydrin. The fraction with the same R_F as alanine (fraction 2 in Fig. 3) was cut out and eluted with water; evaporation of the eluate gave a brown syrup (19·8 mg.). The syrup was dissolved in water (0·15 ml.) and some contaminating solid spun down. A portion (10 μl.) of the supernatant was diluted with 10% (v/v) propan-2-ol (40 μl.) and this solution examined by paper chromatography in butanol–acetic acid and electrophoresis at pH 4 and pH 2. These tests showed a strong ninhydrin-positive spot, not separated from ophthalmic acid in mixed chromatograms; a faint spot was also seen, travelling further in paper electrophoresis at both pH values.

The bulk of the solution of ophthalmic acid was treated with 0·3M-copper acetate (0·1 ml.) and kept in the refrigerator overnight. The crystalline copper salt of ophthalmic acid separated (6 mg.).

In another experiment the anion-exchange resin Amberlite IR-45 was used in the acetate form. The rest of the isolation was carried out as described above and the copper salt of ophthalmic acid (4 mg.) isolated.

Conversion of the copper salt of ophthalmic acid into the free tripeptide. A warm solution of 8-hydroxyquinoline in $CHCl_3$ (5 ml. of 0·1%, w/v) was added to the powdered copper salt (4 mg.) suspended in water (0·5 ml.); the $CHCl_3$ layer was separated and the extraction repeated until the $CHCl_3$ layer was colourless. Each lot of the $CHCl_3$ solution was extracted successively with water (0·3 ml.); the combined aqueous layers were then extracted three times with $CHCl_3$, concentrated to a volume of about 0·05 ml., and cautiously diluted with acetone till turbid. After a short time, the initial precipitate was spun down; crystalline ophthalmic acid slowly separated from the supernatant.

Action of carboxypeptidase on ophthalmic acid. Potassium carbonate (2·4 mg.) was added to carboxypeptidase (0·3 mg., Armour Laboratories) in water (0·03 ml.); as soon as the enzyme had dissolved, ophthalmic acid (approximately 0·2 mg.) was added, and the pH of the solution lowered by passing CO_2 over the surface. The reaction was followed by paper chromatography in butanol–acetic acid. After 8 hr. decomposition of ophthalmic acid was nearly complete, and a strong spot with the same R_F (and colour) as glycine had appeared; the other product had the same R_F as γ-L-glutamyl-DL-α-amino-*n*-butyric acid, applied as a superimposed spot. It was observed that the R_F of this dipeptide was lowered by the other constituents of the reaction mixture. An authentic specimen of the dipeptide was prepared by condensing benzyloxycarbonyl-γ-L-glutamyl azide with α-amino-*n*-butyric acid, followed by hydrogenation (S. G. Waley, paper in preparation).

Norophthalmic acid. The ninhydrin-positive band (fraction 3 in Fig. 3) travelling just behind ophthalmic acid was eluted and the eluate evaporated. The experiment using Amberlite IR-4B gave 6·8 mg., and that using Amberlite IR-45 gave 5·8 mg.; the samples were united. Further fractionation was necessary, as ophthalmic acid was still present. The solution was spread as a band, 30 cm. long, on the washed no. 3 paper, and run for 36 hr. in butanol–acetic acid. Strips cut out and developed with ninhydrin showed two bands: the faster was ophthalmic acid and the slower one the new peptide.

Table 1. *Structure of norophthalmic acid*

The peptide was treated with 1-fluoro-2:4-dinitrobenzene in aqueous ethanol containing triethylamine carbonate, and the dinitrophenyl derivative isolated, by the general method previously described (Waley, 1956).

Amino acid	Molar ratio amino acid:Ala	Strength of amino acid in hydrolysate of dinitrophenyl-norophthalmic acid	2:4-Dinitrophenyl amino acid identified
Gly	1·2	+ + +	—
Glu	1·3	–	Glu
Ala	1·0	+ + +	—

Amino acid analysis carried out, as described in the *General* section, on a portion of the hydrolysate gave the results shown in Table 1; glutamic acid was also found to be the *N*-terminal amino acid.

The reaction with carboxypeptidase was carried out as described for ophthalmic acid. The amino acid liberated was isolated by the method of Thompson (1952) and identified by paper chromatography in butan-2-ol–aq. 3% (w/v) NH_3 soln. (5:2, v/v) (Hausmann, 1952) as glycine.

The glutamic acid residue in norophthalmic acid is believed to be γ-linked (on the basis of the electrophoretic mobility; this evidence will be discussed in detail in a later paper).

DISCUSSION

Isolation of ophthalmic acid

Both the cation-exchange resin (Zeo-Karb 225) and the anion-exchange resins (Amberlite IR-4B or IR-45) could be used to adsorb ophthalmic acid directly from the disintegrated lens, although there is about a thousand times as much protein present as there is ophthalmic acid. Acidic amino acids and peptides were isolated by the successive use of the anion-exchange resin (as its acetate) and the cation-exchange resin (in the acid form) (Fig. 3); the first adsorbs acidic species and the second amino compounds, provided that they do not contain an acidic group comparable in strength with that of the acidic resin. A detailed discussion of the relationship between the ionic charge of a solute and its adsorption by ion-exchange resins has been given by Partridge & Brimley (1952). The main ninhydrin-positive constituents of the fraction obtained by the use of these resins were glutamic acid, glutathione and ophthalmic acid, and this mixture can be directly resolved by paper chromatography. This fraction, then, resembles the fraction L-II-2 previously obtained by electrophoresis (Waley, 1956); this was expected, as both methods of fractionation depend mainly on the net ionic charge.

The ophthalmic acid obtained from the calf lens was obtained in a crystalline form, and comparison with synthetic material established the structure (Waley, 1957); a detailed account of the synthesis will be published later. Since the peptide is more readily available by synthesis than by isolation from calf lens, no investigation has been made of the losses incurred during the isolation.

Norophthalmic acid

One of the minor constituents of the mixture of acidic ampholytes separated by paper chromatography has also been identified. Evidence from degradation (with the same methods as were applied to ophthalmic acid) leads to the structure γ-glutamylalanylglycine; thus the peptide is a lower homologue of ophthalmic acid, and has been named norophthalmic acid; it was also detected in the earlier work (Waley, 1956; fraction L-II-2-Ox-ε-2). This structure is confirmed by the observation that norophthalmic acid from the lens is chromatographically indistinguishable from γ-glutamylalanylglycine obtained by the action of Raney nickel on glutathione; this synthesis has also been described by Kermack & Matheson (1957*a*). The optically inactive tripeptide, γ-DL-glutamyl-DL-alanylglycine, was found to act as an inhibitor of glutathione in the glyoxalase reaction (Kermack & Matheson, 1957*b*); the configuration of the norophthalmic acid from the lens is unknown, but it seems likely that both the glutamic acid and alanine residues will have the L-configuration; so presumably the norophthalmic acid in the lens is a somewhat more active inhibitor than the material studied by Kermack & Matheson. The effect of ophthalmic acid itself in the glyoxalase reaction will be investigated soon.

Occurrence of γ-glutamyl peptides

The γ-linked peptides of glutamic acid at present known are a diverse class of metabolites; bacterial polyglutamic acid (Waley, 1955) is of restricted occurrence; a peptide, believed to be γ-glutamylalanine, has been found in pea seeds (Virtanen & Berg, 1954); the pteroylglutamic acids and glutathione are widely distributed. Of these compounds, it is glutathione that ophthalmic acid and norophthalmic acid resemble most closely, and it will be interesting to see whether they are at all widely distributed; ophthalmic acid has not been detected in the liver or erythrocytes of the calf (Waley, 1956), and there are several general reports on the failure to detect peptides (other than glutathione) in tissues (Askonas, Campbell, Godin & Work, 1955; Christensen & Riggs, 1953). As ophthalmic acid has the same R_F values as alanine, and norophthalmic acid as glycine, in both phenol–ammonia and butanol–acetic acid, paper chromatography of unfractionated tissue extracts will not readily reveal these peptides.

SUMMARY

1. The tripeptide, ophthalmic acid, has been isolated in the crystalline state from calf lens.

2. The isolation depends on the use of ion-exchange resins as molecular sieves: the peptide is selectively adsorbed from dilute solution without interference from proteins. Further fractionation has been effected with ion-exchange resins and paper chromatography.

3. Another tripeptide (norophthalmic acid) has been identified: it is γ-glutamylalanylglycine.

I should like to thank my colleagues at this Laboratory for advice and encouragement, Miss Joyce Blackwell for the collection of calves' eyes, the staffs of the Oxford Co-operative and Eastwyck Farm Slaughterhouses for their co-operation, the National Council to Combat Blindness, Inc., New York, for a grant for chromatographic equipment, and The Distillers Co. Ltd. for a gift of glutathione.

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